

The present application is a continuation-in-part of U.S. Patent application 08/440,421 – filed May 15, 1995 which is a CIP of U.S. Patent application 08/323,460 filed October 14, 1994 both of which applications are continuations in part of U.S.S.N. 08/049,254, filed April 15, 1993, and now U.S. Patent No. 5,405,941. The present application is also a continuation-in-part of U.S.S.N. 08/410,602, filed March 24, 1995, and of U.S.S.N. 08/472,934, filed June 6, 1995. The above-referenced patents and patent applications are incorporated herein by this reference in their entirety.

This invention was made in part with government support under USPHS Grant DK37871 and USPHS Grant GM30324, both awarded by the National Institutes of Health. The government has certain rights to this invention.

This invention relates to isolated nucleic acid molecules encoding MEKK proteins, substantially pure MEKK proteins, and products and methods for regulating signal transduction in a cell.

Mitogen-activated protein kinase (MAPKs) (also called extracellular signal-regulated kinases or ERKs) are rapidly activated in response to ligand binding by both growth factor receptors that are tyrosine kinases (such as the epidermal growth factor (EGF) receptor) and receptors that are coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) such as the thrombin receptor. In addition, receptors like the T cell (TCR) and B cell (BCR) receptors are non-covalently associated with src family tyrosine kinases which activate MAPK pathways. Specific cytokines like tumor necrosis factor (TNF α) can also regulate MAPK pathways. The MAPKs appear to integrate multiple intracellular signals transmitted by various second messengers. MAPKs phosphorylate and regulate the activity of enzymes and transcription factors including the EGF receptor, Rsk 90, phospholipase A₂, c-Myc, c-Jun and Elk-1/TCF. Although the rapid activation of MAPKs by receptors that are tyrosine kinases is dependent on Ras, G protein-mediated activation of MAPK appears to occur through pathways dependent and independent of Ras.

Certain biological functions, such as growth and differentiation, are tightly regulated by signal transduction pathways within cells. Signal transduction pathways maintain the balanced steady state functioning of a cell. Disease states can arise when signal transduction in a cell breaks down, thereby removing the tight control that typically exists over cellular functions. For example, tumors develop when regulation of cell growth is disrupted enabling a clone of cells to expand indefinitely. Because signal transduction networks regulate a multitude of cellular functions depending upon the cell type, a wide variety of diseases can result from abnormalities in such networks. Devastating diseases such as cancer, autoimmune diseases, allergic reactions, inflammation, neurological disorders and hormone-related diseases can result from abnormal signal transduction.

The present invention relates to a substantially pure MEKK protein capable of regulating a MEK kinase dependent pathway. In certain embodiments a MEK kinase comprises a catalytic domain and is capable of phosphorylating MKK proteins. In preferred embodiments the MEKK substrate is selected from the group of MAP kinase kinases consisting of MEKK1, MKK2, (also called MEK1 and MEK2 respectively) MKK3, or MKK4 (also called JNKK1 and JNKK2 or SEK respectively). The present invention

includes a substantially pure MEKK protein capable of regulating signals initiated from a growth factor receptor on the surface of a cell by regulating the activity of MAPK protein. Exemplary MAP kinases include p42, p44, ERK1, ERK2, JNK1, JNK2, or p38 SAPK. In preferred embodiments a MEK kinase can activate at least one of the group c myc, cJun, cPLA2, Rsk 90, TCF, Elk-1, or ATF-2.

In certain embodiments the MEKK protein of the present invention is regulates the activity of a MAPK protein independently of Raf. In preferred embodiments the MEKK proteins described herein are capable of binding members of the Ras superfamily. Exemplary polypeptides which bind to MEKK proteins include Ras, Rac/Cdc42, or Rho.

In particular, the substantially pure MEKK proteins of the present invention comprise at least a portion of an amino acid sequence shown in one of SEQ ID Nos: 2, 4, 6, 8, 10, 12, or 14. In other embodiments, proteins at least 50% homologous, at least 75% homologous, preferably at least 85% homologous, or more preferably 95% homologous to one of SEQ ID Nos: 2, 4, 6, 8, 10, 12, or 14 are also contemplated.

In certain embodiments MEKK proteins have homology to the kinase catalytic domain of one of SEQ ID Nos: 2, 4, 6, 8, 10, 12, or 14. In other embodiments proteins having at least 50% homology, at least 75% homology, preferably at least 85% homology, or more preferably at least 95% homology to the kinase catalytic domain of one of SEQ ID Nos: 2, 4, 6, 8, 10, 12, or 14 are contemplated. In more preferred embodiments the kinase domain of a MEKK protein is capable of phosphorylating a MAP kinase kinase protein and binding to a member of the ras superfamily, such as ras or rac or cdc42 protein.

In another embodiment the MEKK protein of the present invention comprises a NH2 regulatory domain represented in one of SEQ ID Nos: 2, 4, 6, 8, 10, 12, or 14. In other embodiments MEKK proteins which comprise regions of at least 50% homology, at least 75% homology, preferably 85% homology, or more preferably at least 95% homology to the NH2 regulatory domain of one of SEQ ID Nos 2, 4, 6, 8, 10, 12, or 14 are contemplated.

In a further embodiment MEKK proteins which have molecular weights ranging from 60 to 190 are contemplated. Preferred molecular weights are 98 kD for MEKK1, 69.5kD for MEKK2, 71kD for MEKK3, and 95-98 kD for MEKK 4. In other embodiments MEKK 4 migrates with an apparent molecular weight of 185 kD.

MEKK proteins of the present invention lack an SH2 or SH3 domain. In preferred embodiments exemplary MEKK proteins comprise a proline rich SH3 binding motif. In certain embodiments, MEKK proteins of the instant invention comprise a Pleckstrin homology domain.

In a particularly preferred embodiment, exemplary MEKK proteins can competitively inhibit the activity of a MEKK designated in one or more of SEQ ID Nos: 2, 4, 6, 8, 10, or 12, or 14.

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In another embodiment nucleic acids at least 50%, at least 75%, more preferably at least 85%, or most preferably 95% homologous to one of SEQ ID Nos: 1, 3, 5, 7, 9, 11, or 13 are also contemplated.

In another embodiment the nucleic acid of the present invention encodes a polypeptide, wherein said polypeptide i) phosphorylates a MAP kinase kinase protein and ii) binds to a ras superfamily protein. In certain embodiments the ras superfamily member is ras and said binding is mediated by the carboxy terminus of said polypeptide. In another embodiment the nucleic acid encodes a protein with a cdc42/rac binding site.

In another embodiment the nucleic acid of the present invention encodes a polypeptide which comprises a MKK consensus binding site. In another embodiment the nucleic acid of the present invention encodes a polypeptide which comprises a proline rich SH3 binding motif.

In another embodiment the nucleic acid of the present invention is capable of hybridizing under stringent conditions to a nucleic acid probe having a sequence represented by at least 60 consecutive nucleotides of sense or antisense of one or more of SEQ ID Nos: 1, 3, 5, 7, 9, 11, or 13. Oligonucleotide probes which hybridize to one of SEQ ID Nos: 1, 3, 5, 7, 9, 11, or 13 are also contemplated.

Another aspect of the present invention includes a recombinant molecule, comprising a nucleic acid molecule capable of hybridizing under stringent conditions with a nucleic acid sequence including SEQ ID Nos: 1, 3, 5, 7, 9, 11, or 13 in which the nucleic acid molecule is operatively linked to an expression vector.

In another embodiment a nucleic acid of the present invention is operably linked to a transcriptional regulatory sequence and said gene construct is deliverable to a cell and causes the cell to be transfected with said gene construct.

Yet another aspect of the present invention is a recombinant cell transformed with a recombinant molecule, comprising a nucleic acid molecule operatively linked to an expression vector, the nucleic acid molecule comprising a nucleic acid sequence capable of hybridizing under stringent conditions with a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, or 13.

In another embodiment the present invention comprises a host cell transfected with the expression vector comprising one of SEQ ID Nos: 1, 3, 5, 7, 9, 11, or 13. Another embodiment of the present invention comprises a method for producing recombinant MEKK polypeptide by culturing a host cell transfected with such an expression vector.

Also contemplated by the present invention are transgenic animals having cells which harbor a transgene encoding a MEKK polypeptide or in which a gene for a MEKK is disrupted.

One embodiment of the invention provides for drug screening assays that can be used to identify compounds which inhibit the interaction of MEKK with a MEKK binding protein, said binding protein including a substrate or upstream activator of MEKK as described herein. The invention further contemplates the development of peptides or mimetics or

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nucleic acids which can block MEKK activation in a similar manner. In a preferred embodiment a peptide which blocks the interaction of a MEKK protein with Rac or Cdc42 is provided. In a further preferred embodiment a peptide which blocks the interaction of a MEKK protein with Ras is also provided.

The present invention also includes a method for regulating the homeostasis of a cell comprising regulating the activity of a MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in the cell. In particular, the method comprises regulating the apoptosis of the cell. Such a method is useful for the treatment of a medical disorder. In particular, the method is useful for inhibiting tumorigenesis and autoimmunity.

According to the present invention, the method for treatment of a disease, comprises administering to a patient an effective amount of a therapeutic compound comprising at least one regulatory molecule including a molecule capable of decreasing the activity of a Raf-dependent pathway, a molecule capable of increasing the activity of a MEKK-dependent pathway, and combinations thereof, in which the effective amount comprises an amount which results in the depletion of harmful cells involved in the disease.

Also included in the present invention is a therapeutic compound capable of regulating the activity of a MEKK-dependent pathway in a cell identified by a process, comprising: (a) contacting a cell with a putative regulatory molecule; and (b) determining the ability of the putative regulatory compound to regulate the activity of a MEKK-dependent pathway in the cell by measuring the activation of at least one member of said MEKK-dependent pathway.

One embodiment of the present invention includes a substantially pure protein, in which the protein is isolated using an antibody capable of selectively binding to a MEKK protein capable of phosphorylating mammalian MKK proteins and capable of regulating the activity of MAPK proteins independent of Raf protein, the antibody capable of being produced by a method comprising: (a) administering to an animal an effective amount of a substantially pure MEKK protein of the present invention; and (b) recovering an antibody capable of selectively binding to the MEKK protein.

Another embodiment of the present invention includes an isolated antibody capable of selectively binding to a MEKK protein, the antibody capable of being produced by a method comprising administering to an animal an effective amount of a substantially pure protein of the present invention, and recovering an antibody capable of selectively binding to the MEKK protein. Also contemplated by the present invention is a MEKK polypeptide bound by an antibody which specifically binds to a MEKK protein shown in one of SEQ ID Nos: 2, 4, 6, 8, 10, or 12.

This invention further relates to biological responses modulated by the MAPK pathway, which is regulated by signaling through interactions of Ras protein and MEK kinase

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The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Brief Description Of The Figures

Figure 1 is a schematic representation of the signal pathways of vertebrates and yeast.

Figure 2 is a schematic representation of the dual MEKK and Raf pathways divergent from Ras protein pathway.

Figure 3 shows the activation of MAPK in COS cells transfected with MEKK.

Figure 4 shows the activation and phosphorylation of MEK in COS cells transfected with MEKK.

Figure 5 shows the relative ability of immunoprecipitated MEKK and Raf-B to phosphorylate kinase inactive MEK-1.

Figure 6 shows a time course of EGF-stimulated MEKK and Raf-B activation.

Figure 7 shows that the immunodepletion of Raf-B from MEKK immunoprecipitates has no effect on MEKK activity.

Figure 8 shows that the immunodepletion of Raf-B from MEKK immunoprecipitates decreases Raf-B activity.

Figure 9 shows inhibition of MEKK and Raf-B activation by dominant negative N¹⁷RAS expression.

Figure 10 shows inhibition of EGF activation of MEKK by forskolin.

Figure 11 shows improved MEKK activity by truncated MEKK molecules.

Figure 12 shows JNK activation by MEKK protein.

Figure 13 shows regulation of c-Myc controlled transcription and not CREB controlled transcription by MEKK protein.

Figure 14 is a schematic representation of MEKK regulation of c-Myc controlled transcription.

Figure 15 shows wild type Swiss 3T3 cells transfected with pCMV5BXB_{Raf} or pCMV5 without a cDNA insert in the presence of expression plasmids encoding Gal4/Elk-1 and Gal4-TK-luciferase. Cells were lysed and assayed for luciferase activity 48 hours post-transfection.

Figure 16. Induction of MEKKCOOH expression by IPTG in Swiss 3T3 cells increases the number of condensed cells and stimulates c-Myc transactivation. In panel A, cells were incubated in the presence or absence of 5 mM IPTG for forty eight hours. Cells were stained with acrodine orange and condensed cells quantitated per 1000 cells counted per coverslip. In panel B Swiss 3T3 cells with inducible MEKKCOOH were incubated in the presence or absence of IPTG. The indicated cells were then exposed to UV-C irradiation and then fixed and stained with propidium iodide. The percentage of apoptotic cells was enumerated.

Figure 17 shows that MEKKCOOH stimulates JNK/SAPK α but did not activate ERK (p42/44 MAPK) or p38Hog1. Induction of MEKKCOOH does not activate ERK or p38, whereas PDGF or sorbitol, (used as positive controls) do. Activation of the cells with PDGF or sorbitol activated ERK and p38/Hog1 as a control.

Figure 18 shows that induction of MEKKCOOH expression did not significantly increase Gal4/Jun transactivation (left panel). Transient transfection of MEKKCOOH resulted in increased Gal4/Jun transactivation in the MEKK2 Swiss 3T3 clone (right panel).

Figure 19 shows that competitive inhibitory JNK/SAPK(APF) attenuates Gal4/Jun but not Gal4/myc activation. The results are representative of three independent experiments where a three-fold excess of JNK/SAPK(APF) inhibited approximately 65% of Gal4/Jun activation with no effect on Gal4/myc activation.

Figure 20 shows the induction of apoptosis in L929 cells expressing MEKKCOOH domain by TNF.

Figure 21 shows similar stimulation of MAPK activity by MEKK protein and Raf protein.

Figure 22 is a graph illustrating the ability of various MEKK proteins, and fragments thereof, to activate a JNK activity.

Figure 23 is a graph illustrating the ability of various MEKK proteins, and fragments thereof, to activate ERK1 and ERK2.

Figure 24¹ This figure shows that TNF induces apoptosis in L929 cells and that this effect is blocked by bFGF. In panel A cells were treated with the indicated concentrations of TNF α for 15 hours and were assayed for uptake of neutral red. In panel B cells were untreated (solid bars), treated with 0.5 ng/ml bFGF (dotted bars) or 5.0 ng/ml bFGF (hatched bars) and the indicated concentrations of TNF α for 18 hours. Cell viability was assessed by neutral red assay.

Figure 25 shows the activation of JNK and MAPK in L929 cells. In panel A cells were treated for 10 minutes with the indicated concentration of TNF α . JNK activation was measured using a solid phase kinase assay resulting in phosphorylation of GST-Jun. In panel C the time course of MAPK activation is shown. MAPK was isolated from cell lysates on DEAE sephacel columns and MAPK activation was measured by phosphorylation of the EGFR peptide substrate. Panel C depicts the concentration curve of MAPK activation by TNF α . Cells were treated with the indicated concentration of TNF α and MAPK was assayed.

Figure 26 depicts the activation of MAPK by bFGF in L929 cells. Serum starved L929 cells were stimulated for 10 min with the indicated concentration of bFGF.

Figure 27 shows that bFGF does not inhibit TNF α stimulation of JNK activity. In panel A serum starved L929 cells were treated as indicated. Radiolabel incorporated into

GST-Jun is expressed in arbitrary phosphorimaging units. In panel B cells were stimulated as indicated and assayed for MAPK activity.

Figure 28 shows the effect of dominant negative N17 Ras or constitutively active V12 Ras on MAPK and JNK activities. In panel A cells were uninduced (-) or induced (+) to express N17 Ras by overnight treatment with 5 mM IPTG. The cells were unstimulated(-) or stimulated(+) for 10 min with 0.5ng/ml bFGF. MAPK activity was assayed. In panel B 41.LAC1 or V12 Ras cells were induced with IPTG, stimulated as indicated and analyzed for MAPK activation.

Figure 29- shows the effect of N17 Ras on TNF α killing and bFGF protection. Ras expression was induced with 5mM IPTG for 10 hours and cells were subsequently treated with 5 ng/ml TNF α in the presence or absence of 0.5 ng/ml bFGF for 16 hours. Cells were fixed and stained with propidium iodide. The percentage of apoptotic cells was calculated. Solid bars represent cells induced with IPTG; hatched bars, induced with IPTG and treated with TNF α ; checked bars, induced with IPTG and treated with TNF α and bFGF.

Figure 30 shows the inhibition of MAPK activity and elimination of the bFGF protective effect of treatment with the MEK-1 inhibitor PD #098059. In panel A serum starved L929 cells were untreated or treated for 1 hour at 37°C with the MEK-1 inhibitor (PD) and then unstimulated or stimulated with bFGF. MAPK activity was measured. In panel B L929 cells were untreated or treated for 1 hour at 37°C with PD and then were untreated or treated with TNF α alone or in combination with bFGF for 18 hours. Cell viability was assessed by neutral red assay.

Detailed Description Of The Invention

Through a series of inducible and reversible protein-protein interactions and phosphorylation-mediated enzymatic activities, regulatory proteins are recruited to relay signals throughout the cell. Such interactions are involved in all stages of the intracellular signal transduction process - at the plasma membrane, where the signal is initiated; in the cytoplasm, where the signals are disseminated to different cellular locations; and in the nucleus, where other proteins involved in transcriptional control form complexes to regulate transcription of particular genes. The structural nature of protein interactions and control of enzymatic activities in signal transduction is emerging through the identification of the individual proteins that participate in each signal transduction pathway, the elucidation of the temporal order in which these proteins interact, and the definition of the sites of contact between the proteins. The understanding gained in intracellular signaling pathways of cells will be advantageous in developing the next generation of pharmaceuticals. In particular, the pleiotropic richness of intracellular signaling pathways

in cells represents a means for developing more selective pharmacological activity in a therapeutic agent than may be possible in the present generation of drugs.

The present invention concerns the discovery of a family of novel mitogen ERK kinase kinase proteins (referred to herein as "MEK kinases", "MEKKs" or "MEKK proteins") which function in intracellular signal transduction pathways in a variety of cells, and accordingly have a role in determining cell/tissue fate and maintenance. The family of MEKK genes or gene products provided by the present invention apparently consists of at least six different members (MEKK 4.2 is a splicing variant of MEKK4.1 and MEKK 2.2 is a sequencing variant of MEKK2) with ample evidence indicating that yet other members of the family exist.

A salient feature of the MEKK gene products deriving from this discovery not only implicates these proteins in intracellular signaling, but also strongly suggests that the diversity of the MEKK family is important to providing a diversity of responses to different environmental cues. That is, the ability of a cell to respond to a particular growth factor, morphogen, or even stress cue, and the type of response the cell undergoes is dependent at least in part upon which MEKK gene products are expressed in the cell and/or engaged by signals propagated upstream of the kinase.

Still another important feature of the present invention is the discovery of the involvement of MEKK proteins in certain apoptotic pathways.

Accordingly, certain aspects of the present invention relate to nucleic acids encoding vertebrate MEKK proteins, the MEKK proteins themselves, antibodies immunoreactive with MEKK proteins, and preparations of such compositions. Moreover, the present invention provides diagnostic and therapeutic assays and reagents for detecting and treating disorders involving, for example, aberrant expression or activation of the MEKK gene products. In addition, drug discovery assays are provided for identifying agents which can modulate the biological function of MEKK proteins, such as by altering the binding of the protein to either downstream or upstream elements in a signal transduction pathway, or which inhibit the kinase activity of the MEKK protein. Such agents can be useful therapeutically to alter the growth and/or differentiation of a cell. Other aspects of the invention are described below or will be apparent to those skilled in the art in light of the present disclosure.

Initial cloning of a member of the mammalian MEKK family was accomplished using primers based on sequences for the yeast protein kinases Byr2 (from *S pombe*) and Ste11 (from *S. cerevisiae*). Using the sequence obtained for the mammalian MEKK cDNA, other MEKK transcripts have been detected and several subsequently cloned to reveal a family of mammalian MEKK proteins.

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Table 1
Guide to MEKK sequences in Sequence Listing

	Nucleotide	Amino Acid
MEKK1.1	SEQ ID No. 1	SEQ ID No. 2
MEKK1.2	SEQ ID No. 3	SEQ ID No. 4
MEKK2.1	SEQ ID No. 5	SEQ ID No. 6
MEKK2.2	SEQ ID No. 7	SEQ ID No. 8
MEKK3	SEQ ID No. 9	SEQ ID No. 10
MEKK4.1	SEQ ID No. 11	SEQ ID No. 12
MEKK4.2	SEQ ID No. 13	SEQ ID No. 14

The foregoing SEQ ID NO's represent sequences deduced according to methods disclosed in the Examples. It should be noted that since nucleic acid and amino acid sequencing technology is not entirely error-free, the foregoing SEQ ID NO's, at best, represent apparent nucleic acid and amino acid sequences of MEKK proteins of the present invention. For convenience, we will use the term MEKK1 to refer to both MEKK1.1 and MEKK 1.2, MEKK 2 to refer to both MEKK2.1 and MEKK 2.2, and MEKK4 to refer to both MEKK4.1 and MEKK 4.2 herein.

The primary sequence of the MEKK proteins suggests two functional domains, an amino-terminal moiety rich in serine and threonine that apparently serves a regulatory role, and a carboxy-terminal protein kinase catalytic domain. The catalytic domain of, for example, MEKK1 shows approximately 35 percent identity with the amino acid sequences of the catalytic domains of Byr2 and Ste11. The amino-terminal moieties of each of the mammalian MEKKs show little similarity with Ste11 or Byr2.

Furthermore, the MEKK family is apparently encoded by several genes, at least some of which are able to produce different transcripts by differential splicing. For example, the divergence in sequence amongst the catalytic domains of each of MEKK1 to MEKK4 indicated that separate genomic genes encode each paralog. However, MEKK2 and MEKK4 genes can give rise to at least two different transcripts, presumably by differential splicing. Expression data suggests that MEKKs 1-4 are ubiquitously expressed.

By use of overexpression and/or constitutively activated MEKKs, a variety of cellular substrates for each MEKK protein have been identified. In general, the proteins of the MAP kinase kinases (MEK) family are each targets for one or more of the MEKKs. Moreover, the data set out below demonstrate that MEKK-dependent signal propagation can result in the phosphorylation/activation of members of the MAP kinase family, such as p42MAPK, p44MAPK, p38MAPK, and the Jun NH₂-terminal kinases (JNKs).

Certain of the MEKK proteins have been shown to be activated, e.g., as kinases, in response to growth factors and cytokines (such as TNF α and chemoattractants like FMLP

and IL-8) and other environmental cues, including stress, as well as expression of activated Ras or other members of the Ras Superfamily, including Rac and Cdc 42. It is demonstrated below that the kinase domain of at least MEKK1 binds to activated Ras in a GTP-dependent manner, implicating that interaction as a potential therapeutic target. Moreover, a Ras effector domain peptide blocks the binding of the MEKK catalytic domain with the GTP-bound form of Ras. In addition, it is shown in the appended Examples that MEKK4 binds to Rac, a low molecular weight GTP binding protein of the Ras superfamily. The sequence of MEKK4 which binds to Cdc42 and Rac has been identified. This sequence IIGQVCDTPKSYDNVMHVGLR occurs around residue 1306-1326 of MEKK4.2 or 599-619 of MEKK4 and peptides from this region can be used to block the binding of the MEKK catalytic domain with Cdc42 and Rac.

Yet another set of experimental data provided in the appended examples indicates that activation of certain MEKK pathways can lead to apoptosis. The integration of signal transduction pathways regulated by growth factor and cytokine receptors commits a cell either to proliferation or apoptosis (Sumimoto, S.L. et al. (1994) J. Immunol. 153:2488-2496). Specific cytokines and stresses to cells, such as DNA damage, appear to preferentially activate the JNK/SAPK pathway which leads to apoptosis. Several checkpoints exist in the pathways leading to apoptosis that involve proteins such as Bcl2 and p58, which can both inhibit apoptosis. The MEKK proteins are therefore, important to the dynamic balance between growth factor-activated ERK and stress-activated JNK/p38 pathways and accordingly important in determining whether a cell survives or undergoes apoptosis. To date candidate molecules involved in signaling apoptosis include ceramide, Ras, Rho, c-myc, c-Jun, and the proteins associated with the TNF α receptor and Fas.

One aspect of the present invention relates to isolated MEKK proteins. As used herein protein, peptide, and polypeptide are meant to be synonymous. According to the present invention, an isolated protein is a protein that has been removed from its natural milieu. It will be understood that "isolated", with respect to MEKK polypeptides, is meant to include formulations of the polypeptides which are isolated from, or otherwise substantially free of other cellular proteins ("contaminating proteins"), especially other cellular signal transduction factors, normally associated with the MEKK polypeptide. Thus, isolated MEKK protein preparations include preparations having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). Functional forms of the subject MEKK polypeptides can be prepared, for the first time, as purified preparations by using a cloned gene as described herein. Alternatively, the subject MEKK polypeptides can be isolated by affinity purification using, for example, a catalytically inactive MEK. "Isolated" does not encompass either

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myristoylation, p.

A homologue of a MEKK protein is a protein having an amino acid sequence that is sufficiently similar to a natural MEKK protein amino acid sequence that a nucleic acid sequence encoding the homologue is capable of hybridizing under stringent conditions to (i.e., with) a nucleic acid sequence encoding the natural MEKK protein amino acid sequence. As used herein, stringent hybridization conditions refer to standard hybridization conditions under which nucleic acid molecules, including oligonucleotides, are used to identify similar nucleic acid molecules. Such standard conditions are disclosed, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989. A homologue of a MEKK protein also includes a protein having an amino acid sequence that is sufficiently cross-reactive such that the homologue has the ability to elicit an immune response against at least one epitope of a naturally-occurring MEKK protein.

The minimal size of a protein homologue of the present invention is a size sufficient to be encoded by a nucleic acid molecule capable of forming a stable hybrid with the

complementary sequence of a nucleic acid molecule encoding the corresponding natural protein. As such, the size of the nucleic acid molecule encoding such a protein homologue is dependent on nucleic acid composition, percent homology between the nucleic acid molecule and complementary sequence, as well as upon hybridization conditions per se (e.g., temperature, salt concentration, and formamide concentration). The minimal size of such nucleic acid molecules is typically at least about 12 to about 15 nucleotides in length if the nucleic acid molecules are GC-rich and at least about 15 to about 17 bases in length if they are AT-rich. As such, the minimal size of a nucleic acid molecule used to encode a MEKK protein homologue of the present invention is from about 12 to about 18 nucleotides in length. There is no limit, other than a practical limit, on the maximal size of such a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof. Similarly, the minimal size of a MEKK protein homologue of the present invention is from about 4 to about 6 amino acids in length, with preferred sizes depending on whether a full-length, multivalent protein (i.e., fusion protein having more than one domain each of which has a function), or a functional portion of such a protein is desired.

MEKK protein homologues can be the result of allelic variation of a natural gene encoding a MEKK protein. A natural gene refers to the form of the gene found most often in nature. MEKK protein homologues can be produced using techniques known in the art including, but not limited to, direct modifications to a gene encoding a protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis. As will be understood, mutagenesis includes point mutations, as well as deletions and truncations of the MEKK polypeptide sequence. The ability of a MEKK protein homologue to phosphorylate MEK and/or JNKK protein can be tested using techniques known to those skilled in the art. Such techniques include phosphorylation assays described in detail in the Examples section.

With respect to homologues, it will also be possible to modify the structure of the subject MEKK polypeptides for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified polypeptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the MEKK polypeptide described in more detail herein. Such modified peptide can be produced, for instance, by amino acid substitution, deletion, or addition.

In one embodiment, a MEKK protein of the present invention is capable of regulating a MEKK-dependent pathway. According to the present invention, a MEKK-dependent pathway refers generally to a pathway in which a MEKK protein regulates a pathway substantially independent of Raf, though the pathway including the MEKK protein may

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In certain preferred embodiments, the MEKK protein will be involved in a pathway controlling the phosphorylation of a mitogen-activated protein (MAP) kinase. The mammalian MAP kinase family includes, for example, the extracellular signal-regulated protein kinases (ERK1 and ERK2), p42 or p44 MAPKs. In another preferred embodiment the MEKK protein will be involved in the pathway controlling c-Jun NH2-terminal kinases (JNKs, or SAPKs), and the so-called "p38 subgroup" kinases (p38 and Hog-1 kinases). For example, it is contemplated that the MEKK proteins of the present invention interact with, and directly phosphorylate members of the MAP kinase kinase family (MEKs or MKKs), as MEK1, MEK2, MKK1, MKK2, or the stress-activated kinases (SEKs), and the Jun kinase kinases (JNKK1, JNKK2, MKK3, MKK4), or the like.

An exemplary MEKK-dependent pathway includes a pathway involving a MEKK protein and a MKK protein. One of skill in the art can determine whether or not the regulation of a pathway by a MEKK protein is substantially independent of a Raf protein by comparing the ability of a MEKK protein and a Raf protein to regulate the phosphorylation of a downstream member of such pathway using, for example, the general method described in Example 16. For instance, a MEKK protein can regulate a pathway substantially independently of a Raf protein if the MEKK protein induces phosphorylation of a member of the pathway downstream of MEKK (e.g., proteins including JEK, Jun kinase, Jun and/or ATF-2) by an amount significantly greater than that seen when Raf protein is utilized. Raf-1 and B-Raf kinases selectively regulate MEK1 and MEK2 and do not recognize the JNKK proteins, thus Raf proteins appear to be highly selective for the regulation of p42/p44 MAPK pathways. MEKK proteins, in contrast, are capable of regulating both JNK and p42/p44 MAPK pathways.

For example, MEKK induction of phosphorylation of a JNK protein is preferably at least about 10-fold, more preferably at least about 20-fold and even more preferably at least about 30-fold than the phosphorylation of the JNK protein induced when using a Raf protein. If MEKK induction of phosphorylation is similar to Raf protein induction of phosphorylation, then one of skill in the art can conclude that regulation of a pathway by a MEKK protein includes members of a signal transduction pathway that could also include Raf protein. For example, MEKK induction of phosphorylation of MAPK is of a similar magnitude as induction of phosphorylation with Raf protein.

A "Raf-dependent pathway" refers to a signal transduction pathway in which a Raf protein regulates a signal transduction pathway substantially independently of a MEKK protein, and a pathway in which Raf protein regulation converges with common members of a pathway involving MEKK protein. The independence of regulation of a pathway by a Raf

protein from regulation of a pathway by a MEKK protein can be determined using methods similar to those used to determine MEKK independence.

In another embodiment, a MEKK protein is capable of regulating the activity of signal transduction proteins including, but not limited to, mitogen activated ERK kinases (MEKs), mitogen activated protein kinases (MAPKs), transcription control factor (TCF), Ets-like-1 transcription factor (Elk-1), Jun ERK kinases (JNKKs), Jun kinases (JNK; which is equivalent to SAPK), stress activated MAPK proteins, Jun, activating transcription factor-2 (ATF-2) and/or Myc protein. As used herein, the "activity" of a protein can be directly correlated with the phosphorylation state of the protein and/or the ability of the protein to perform a particular function (e.g., phosphorylate another protein or regulate transcription). Preferred MEK proteins regulated by a MEKK protein of the present invention include MEK-1 and/or MEK-2 (MKK1 or MKK2). Preferred MAPK proteins regulated by a MEKK protein of the present invention include p38/Hog-1 MAPK, p42 MAPK and/or p44 MAPK. Preferred stress activated MAPK proteins regulated by a MEKK protein of the present invention include Jun kinase (JNK), stress activated MAPK- α and/or stress activated MAPK- β . A preferred MEKK protein that is capable of activating p42/44 MAPK proteins includes a protein encoded by the nucleic acid sequence represented by SEQ ID NO:9 with a protein having the amino acid sequence represented by SEQ ID NO:10 being more preferred. A preferred MEKK protein that is capable of activating JNK MAPK is encoded by the nucleic acid sequence represented by one of SEQ ID Nos: 5 or 7, with a protein having the amino acid sequence represented by one of SEQ ID Nos: 6 or 8 being more preferred.

A MEKK protein of the present invention is capable of increasing the activity of an MEK protein over basal levels of MEK (i.e., levels found in nature when not stimulated). For example, a MEKK protein is preferably capable of increasing the phosphorylation of an MEK protein (such as MEK1 or MEK2, also known as MKK1 and MKK2 respectively) by at least about 2-fold, more preferably at least about 3-fold, and even more preferably at least about 4-fold over basal levels when measured under conditions described in Example 9. In another embodiment, a preferred MEKK protein is capable of increasing the phosphorylation of a JNKK protein (such as JNKK1 or JNKK2, also known as MKK3 and MKK4 respectively).

A preferred MEKK protein of the present invention is also capable of increasing the activity of an MAPK protein over basal levels of MAPK (i.e., levels found in nature when not stimulated). For example, a MEKK protein of the present invention is preferably capable of increasing MAPK activity at least about 2-fold, more preferably at least about 3-fold, and even more preferably at least about 4-fold over basal activity when measured under the conditions described in Example 3.

Moreover, a MEKK protein of the present invention is capable of increasing the activity of a JNK protein. JNK regulates the activity of the transcription factor JUN which is

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In addition, a MEKK protein of the present invention is capable of specific binding to a Ras superfamily protein. In particular, a MEKK protein is capable of binding to a Ras protein that is associated with GTP. According to the present invention, a MEKK protein binds to Ras via the COOH terminal region of the MEKK protein, e.g., a ras-binding domain. A preferred MEKK protein that is capable of binding to Ras or a member of the ras superfamily is encoded by the nucleic acid shown in SEQ ID No:1, 3, 5, 7, 9, 11, or 13 with a protein having the amino acid sequence shown in SEQ ID No:2, 4, 6, 8, 10, 12, or 14 being more preferred. In certain embodiments a MEKK protein is capable of binding to Rac-GTP. A preferred MEKK protein that is capable of binding to Rac or Cdc42 includes a protein encoded by the nucleic acid sequence shown in one of SEQ ID Nos:11 or 13 with a protein having the amino acid sequence represented by one of SEQ ID Nos:12 or 14 being more preferred.

In a preferred embodiment, a MEKK protein of the present invention is capable of phosphorylating a MEK or MKK, Jun kinase kinase (JNKK) and/ or stress activated ERK kinase (SEK), in particular MEK1, MEK2, MKK1, MKK2, MKK3, MKK4, JNKK1, JNKK2, SEK1 and/or SEK2 proteins. As described herein, MEK1 and MEK2 are equivalent to MKK1 and MKK2, respectively. In addition, JNKK1 and JNKK2 are equivalent to MKK3 and MKK4, which are equivalent to SEK1 and SEK2.

A preferred MEKK protein of the present invention is additionally capable of inducing the phosphorylation of a Myc protein, particularly a transcriptional transactivation domain of Myc, in such a manner that the phosphorylated Myc protein is capable of regulating gene transcription. For example, according to Example 17, a MEKK protein of the present invention is preferably capable of inducing luciferase gene transcription by a phosphorylated Myc at least about 25-fold, more preferably at least about 35-fold, and even more preferably at least about 45-fold, over Raf induction when measured under the conditions described in Example 17.

Another aspect of the present invention relates to the ability of a MEKK activity to be stimulated by growth factors including, but not limited to, epidermal growth factor (EGF), neuronal growth factor (NGF), tumor necrosis factor (TNF), C5A, interleukin-8 (IL-8), interleukin-5 (IL-5), monocyte chemotactic protein 1 (MIP1 α), monocyte chemoattractant

Preferably, the activity of certain of the MEKK proteins of the present invention is capable of being stimulated at least 2-fold over basal levels (i.e., levels found in nature when not stimulated), more preferably at least about 4-fold over basal levels and even more preferably at least about 6-fold over basal levels, when a cell producing the MEKK protein is contacted with EGF under the conditions described in Example 3.

On the other hand, as demonstrated below, certain of the MEKK proteins of the present invention are capable of being stimulated by removal of NGF stimulation. MEKK proteins which are stimulated by NGF removal may subsequently cause the activation of one or more p38 kinases and/or JNKs.

In yet another embodiment, a MEKK protein of the present invention is capable of being stimulated at least 0.5-fold over basal levels, more preferably at least about 1-fold over basal levels and even more preferably at least about 2-fold over basal levels by TPA stimulation when a cell producing the MEKK protein is contacted with TPA under the conditions described in Example 9.

TNF is capable of regulating cell death and other functions in different cell types. Another aspect of the present invention relates to the discovery that MEKK stimulation by TNF can be independent of Raf. Similarly, the present invention demonstrates that the kinase activity of certain of the subject MEKK proteins can be stimulated by ultraviolet light (UV) damage of cells. It has been observed that both TNF and UV stimulate MEKK activity without substantially activating Raf. In addition, both UV and TNF activation of MEKK is apparently Ras dependent. In certain embodiments FGF is capable of preventing TNF induced apoptosis.

Another aspect of the present invention is the recognition that a MEKK protein of the present invention is capable of regulating the apoptosis of a cell. As used herein, apoptosis

A preferred MEKK protein of the present invention is capable of inducing the apoptosis of cells, such that the cells have characteristics substantially similar to cytoplasmic shrinkage and/or nuclear condensation as described in the appended Examples. The appended examples also illustrate that TNF and MEKK can synergize to induce apoptosis in cells.

Referring to Figure 2, a MEKK protein of the present invention is capable of being activated by a variety of growth factors and environmental cues capable of activating Ras superfamily protein. In addition, a MEKK protein is capable of activating JNK protein which is also activated by Ras protein, but which is not activated by Raf protein. As such, a MEKK protein of the present invention comprises a protein kinase at a divergence point in a signal transduction pathway initiated by different cell surface receptors. A MEKK protein is also capable of being regulated by TNF protein independent of Raf, thereby indicating an association of MEKK protein to a novel signal transduction pathway which is independent of Ras protein and Raf protein.

Thus, a MEKK protein is capable of performing numerous unique functions independent of or by-passing Raf protein in one or more signal transduction pathways. A MEKK protein is capable of regulating the activity of MEK and/or JNKK activity. As such, a MEKK protein is capable of regulating the activity of members of a signal transduction pathway that does not substantially include Raf activity. Such members include, but are not limited to, JNK, Jun, ATF and Myc protein. In addition, a MEKK protein is capable of regulating the members of a signal transduction pathway that does involve Raf, such members including, but are not limited to, MEK, MAPK and TCF. A MEKK protein of the

present invention is thus capable of regulating the apoptosis of a cell independent of significant involvement by Raf protein.

In addition to the numerous functional characteristics of a MEKK protein, a MEKK protein of the present invention comprises numerous unique structural characteristics. For example, in one embodiment, a MEKK protein of the present invention includes at least one of two different structural domains having particular functional characteristics. Such structural domains include an NH₂-terminal regulatory domain that serves to regulate a second structural domain comprising a COOH-terminal protein kinase catalytic domain that is capable of phosphorylating an MKK protein.

According to the present invention, a MEKK protein of the present invention includes a full-length MEKK protein, as well as at least a portion of a MEKK protein capable of performing at least one of the functions defined above. The phrase "at least a portion of a MEKK protein" refers to a portion of a MEKK protein encoded by a nucleic acid molecule that is capable of hybridizing, under stringent conditions, with a nucleic acid encoding a full-length MEKK protein of the present invention. Preferred portions of MEKK proteins are useful for regulating apoptosis in a cell. Additional preferred portions have activities useful for regulating MEKK kinase activity. Suitable sizes for portions of a MEKK protein of the present invention are as disclosed for MEKK protein homologues of the present invention.

In another embodiment, a MEKK protein of the present invention includes at least a portion of a MEKK protein having molecular weights ranging from about 70 kD to about 250 kD as determined by Tris-glycine SDS-PAGE, preferably using an 8% polyacrylamide SDS gel (SDS-PAGE) and resolved using methods standard in the art. A preferred MEKK protein has a molecular weight ranging from about 65 kD to about 190 kD and even more preferably from about 69 kD to about 98 kD. In particularly preferred embodiments MEKK2 and MEKK3 proteins of the present invention have a molecular weight of about 65-75kD. Preferred MEKK4 proteins have molecular weights about 180-190 kD. Most preferred molecular weights for the subject MEKKs are: >175kD (MEKK1), 69.5 kD (MEKK2 or MEKK2.2), 71 kD (MEKK3), 185kD (MEKK4). It is noted that experimental conditions used when running gels to determine the molecular size of putative MEKK proteins will cause variations in results. Moreover, it has become apparent to the Applicant that, relative to predicted molecular weights, shorter apparently related polypeptides can be observed. Whether these are the result of proteolytic processing, alternative splicing or start codon usage or the like is unclear, but other preferred MEKK1 polypeptides (e.g. MEKK 1.2) have apparent molecular weights of about 95-100 kD; and other preferred MEKK4 polypeptides (e.g., MEKK 4.2) have apparent molecular weights of about 90-100 kD, more preferably 95-98 kD.

homology (identity within comparable regions) with the kinase catalytic domain of a naturally occurring MEKK protein. Another MEKK protein of the present invention also includes at least a portion of a MEKK homologue of the present invention has at least about 50%, more preferably at least about 75%, or most preferably at least about 85% amino acid homology with the NH₂-terminal regulatory domain of a MEKK protein of a naturally occurring MEKK protein.

In certain embodiments MEKK proteins have proline rich sequences that are src homology 3 (SH3) binding motifs. Proline rich regions, specifically the sequence PXXP is thought to be critical in all SH3 ligands (Alexandropoulos and Cheng (1995) *Proc. Natl. Acad. Sci* 92:3110-3114). Preferred MEKK proteins that have proline rich sequences are encoded by nucleic acids shown in one of SEQ ID Nos:3 or 13. In particularly preferred embodiments MEKK proteins comprising SH3 binding motifs are shown in one of SEQ ID Nos:4 or 14. Particularly preferred proline rich sequences are exemplified by the sequences shown in amino acids 26-37 of SEQ ID No:14 or in amino acids 41-51, 70-90, 186-191, 211-219 of SEQ ID No:4.

In other embodiments certain MEKK proteins comprise pleckstrin homology domains. The 'pleckstrin homology' (PH) domain is an approximately 100-residue protein module that is thought to be involved in interactions with GTP-binding proteins (Musacchio et al (1993) *TIBS* 28:343-348). Pleckstrin homology domains are very divergent and do not occupy a specific positions in molecules; alignments of PH domains show six conserved blocks which all contain several conserved hydrophobic residues which are thought to form a folded structure comprising seven to eight β -strands, most likely in one or two β -sheets, and just a single helix (Musacchio et al. *supra*). PH domains have been identified in kinases and also in Vav, Dbl, Bcr, yeast cdc24, Ras-GAP, DM GAP, Ras-GRF, and Sos, all of which are regulators of small GTP-binding proteins. Interestingly, three of the four proteins that have been identified as being capable of binding to SH3 domains (dynamin, 3BP2, and Sos) also contain PH domains (Musacchio et al. *supra*). The PH domain of β adrenergic receptor kinase may be involved in binding to G protein $\beta\gamma$ complexes (Koch et al. (1993) *J. Biol. Chem.* 268:8256-8260). Preferred MEKK proteins that have PH domains are encoded by nucleic acids shown in one of SEQ ID Nos:3 or 13. In particularly preferred embodiments MEKK proteins comprising PH domains are shown in one of SEQ ID Nos:4 or 14. Particularly preferred PH domains are exemplified by the amino acids 262-665 of SEQ ID No:4 or amino acids 233-397 of SEQ ID No:14.

In another embodiment the MEKK proteins of the present invention bind to MKK substrates. Preferred MEKK proteins comprise consensus MKK binding domains as encoded by the nucleic acid sequences shown in one of SEQ ID Nos: 1, 3, 5, 7, 9, 11, or 13. Preferred MKK consensus binding regions are illustrated by amino acids 658-672 of SEQ ID No:2,

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An isolated nucleic acid molecule of the present invention can be obtained from its natural source either as an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with that gene. As used herein, the phrase "at least a portion of" an entity refers to an amount of the entity that is at least sufficient to have the functional aspects of that entity. For example, at least a portion of a nucleic acid sequence, as used herein, is an amount of a nucleic acid sequence capable of forming a stable hybrid with a particular desired gene (e.g., MEKK genes) under stringent hybridization conditions. An isolated nucleic acid molecule of the present invention can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated MEKK protein nucleic acid molecules include natural nucleic acid

Preferred modifications to a MEKK protein nucleic acid molecule of the present invention include truncating a full-length MEKK protein nucleic acid molecule by, for example: deleting at least a portion of a MEKK protein nucleic acid molecule encoding a regulatory domain to produce a constitutively active MEKK protein; deleting at least a portion of a MEKK protein nucleic acid molecule encoding a catalytic domain to produce an inactive MEKK protein; and modifying the MEKK protein to achieve desired inactivation and/or stimulation of the protein, for example, substituting a codon encoding a lysine residue in the catalytic domain (i.e., phosphotransferase domain) with a methionine residue to inactivate the catalytic domain.

Another preferred truncated MEKK nucleic acid molecule encodes a form of a MEKK protein comprising an NH₂-terminal regulatory domain a catalytic domain but lacking a catalytic domain. Preferred regulatory domain truncated MEKK nucleic acid molecules encode amino acid residues from about 1 to about 408 of MEKK 1.1; amino acids 1 to about 1328 of MEKK 1.2; from about 1 to about 360 of MEKK 2.1 or 2.2; from about 1 to about 365 of MEKK 3; from about 1 to about 630 of MEKK 4.1; or from about 1 to about 1337 for MEKK 4.2.

An isolated nucleic acid molecule of the present invention can include a nucleic acid sequence that encodes at least one MEKK protein of the present invention, examples of such proteins being disclosed herein. Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides that comprise the nucleic acid molecule, the two phrases can be used interchangeably. As heretofore disclosed, MEKK proteins of the present invention include, but are not limited to, proteins having full-length MEKK protein coding regions, portions thereof, and other MEKK protein homologues.

As used herein, a MEKK protein gene includes all nucleic acid sequences related to a natural MEKK protein gene such as regulatory regions that control production of a MEKK protein encoded by that gene (including, but not limited to, transcription, translation or post-translation control regions) as well as the coding region itself. A nucleic acid molecule of the present invention can be an isolated natural MEKK protein nucleic acid molecule or a homologue thereof. A nucleic acid molecule of the present invention can include one or more regulatory regions, full-length or partial coding regions, or combinations thereof. The minimal size of a MEKK protein nucleic acid molecule of the present invention is the minimal size capable of forming a stable hybrid under stringent hybridization conditions with a corresponding natural gene.

A MEKK protein nucleic acid molecule homologue can be produced using a number of methods known to those skilled in the art (see, e.g., Sambrook et al., *ibid.*). For example, nucleic acid molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, polymerase chain reaction (PCR) amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof. Nucleic acid molecule homologues can be selected from a mixture of modified nucleic acids by screening for the function of the protein encoded by the nucleic acid (e.g., the ability of a homologue to phosphorylate MEK protein or JNKK protein) and/or by hybridization with isolated MEKK protein nucleic acids under stringent conditions.

One embodiment of the present invention is a MEKK protein nucleic acid molecule capable of encoding at least a portion of a MEKK protein, or a homologue thereof, as described herein. A preferred nucleic acid molecule of the present invention includes, but is not limited to, a nucleic acid molecule that encodes a protein having at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, or homologues thereof. Proteins at least 50%, preferably at least about 75%, more preferably at least about 85%, and most preferably at least about 95% homologous to these sequences are contemplated.

A preferred nucleic acid molecule of the present invention is capable of hybridizing under stringent conditions to a nucleic acid that encodes at least a portion of a MEKK protein, or a homologue thereof. Also preferred is a MEKK protein nucleic acid molecule that includes a nucleic acid sequence having at least about 50% homology, preferably 75% homology, preferably 85% homology, or even more preferably 95% homology with one of SEQ ID No:1, 3, 5, 7, 9, 11, or 13. In other embodiments nucleic acids have 50%, preferably

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Such nucleic acid molecules can be a full-length gene and/or a nucleic acid molecule encoding a full-length protein, a hybrid protein, a fusion protein, a multivalent protein or a truncation fragment. More preferred nucleic acid molecules of the present invention comprise isolated nucleic acid molecules having a nucleic acid sequence as represented by one of SEQ ID Nos: 1, 3, 5, 7, 9, 11, or 13, or nucleic acid molecules capable of hybridizing to said sequences under stringent conditions.

Knowing a nucleic acid molecule of a MEKK protein of the present invention allows one skilled in the art to make copies of that nucleic acid molecule as well as to obtain additional portions of MEKK protein-encoding genes (e.g., nucleic acid molecules that include the translation start site and/or transcription and/or translation control regions), and/or MEKK protein nucleic acid molecule homologues. Knowing a portion of an amino acid sequence of a MEKK protein of the present invention allows one skilled in the art to clone nucleic acid sequences encoding such a MEKK protein.

The present invention also includes nucleic acid molecules that are oligonucleotides capable of hybridizing, under stringent conditions, with complementary regions of other, preferably longer, nucleic acid molecules of the present invention that encode at least a

portion of a MEKK protein, or a homologue thereof. A preferred oligonucleotide is capable of hybridizing, under stringent conditions, with a nucleic acid molecule that is capable of encoding at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID No:14, or homologues thereof. A more preferred oligonucleotide is capable of hybridizing to a nucleic acid molecule having a nucleic acid sequence as represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID No:13 or complements thereof.

Oligonucleotides of the present invention can be RNA, DNA, or derivatives of either. The minimal size of such oligonucleotides is the size required to form a stable hybrid between a given oligonucleotide and the complementary sequence on another nucleic acid molecule of the present invention. Minimal size characteristics of preferred oligonucleotides are at least about 10 nucleotides, preferably at least about 20 nucleotides, more preferably at least about 50 nucleotides and most preferably at least about 60 nucleotides. Larger fragments are also contemplated. The size of the oligonucleotide must also be sufficient for the use of the oligonucleotide in accordance with the present invention. Oligonucleotides of the present invention can be used in a variety of applications including, but not limited to, as probes to identify additional nucleic acid molecules, as primers to amplify or extend nucleic acid molecules or in therapeutic applications to inhibit, for example, expression of MEKK proteins by cells. Such therapeutic applications include the use of such oligonucleotides in, for example, antisense-, triplex formation-, ribozyme- and/or RNA drug-based technologies. The present invention, therefore, includes use of such oligonucleotides and methods to interfere with the production of MEKK proteins. In addition oligonucleotides encoding portions of MEKK proteins which bind to MEKK binding proteins can be used as therapeutics. In other embodiments, the peptides encoded by these nucleic acids are used.

To further illustrate, another aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one or more of the subject MEKK proteins so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is

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Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in *Remington's Pharmaceutical Sciences*, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they specifically bind. Such diagnostic tests are described in further detail below.

Likewise, the antisense constructs of the present invention, by antagonizing the normal biological activity of one of the MEKK proteins, can be used in the manipulation of tissue, e.g. tissue differentiation, both *in vivo* and for *ex vivo* tissue cultures.

Furthermore, the anti-sense techniques (e.g. microinjection of antisense molecules, or transfection with plasmids whose transcripts are anti-sense with regard to a MEKK mRNA or gene sequence) can be used to investigate role of MEKK in disease states, as well as the normal cellular function of MEKK in healthy tissue. Such techniques can be utilized in cell culture, but can also be used in the creation of transgenic animals. The present invention also includes a recombinant vector which includes at least one MEKK protein nucleic acid molecule of the present invention inserted into any vector capable of delivering the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences, for example nucleic acid sequences that are not naturally found adjacent to MEKK protein nucleic acid molecules of the present invention. The vector can be either RNA or DNA, and either prokaryotic or eukaryotic, and is typically a virus or a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulating of MEKK protein nucleic acid molecules of the present invention. One type of recombinant vector, herein referred to as a recombinant molecule and described in more detail below, can be used in the expression of nucleic acid molecules of the present invention. Preferred recombinant vectors are capable of replicating in the transformed cell.

Preferred nucleic acid molecules to insert into a recombinant vector includes a nucleic acid molecule that encodes at least a portion of a MEKK protein, or a homologue thereof. A more preferred nucleic acid molecule to insert into a recombinant vector includes a nucleic acid molecule encoding at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, and/or SEQ ID No:14, or homologues thereof. An even more preferred nucleic acid molecule to insert into a recombinant vector includes a nucleic acid molecule represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, and/or SEQ ID No:13 or complements thereof. In particularly preferred embodiments portions of a MEKK nucleic acid which encodes a MEKK catalytic domain is used. In another particularly preferred embodiment, at least a portion of a nucleic acid which encodes the portion of a MEKK protein which binds to a MEKK substrate or a MEKK regulatory protein is used.

Suitable host cells for transforming a cell can include any cell capable of producing MEKK proteins of the present invention after being transformed with at least one nucleic acid molecule of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule. Suitable host cells of the present invention can include bacterial, fungal (including yeast), insect, animal and plant

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A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules of the present invention operatively linked to an expression vector containing one or more transcription control sequences. The phrase operatively linked refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, insect, animal, and/or plant cells. As such, nucleic acid molecules of the present invention can be operatively linked to expression vectors containing regulatory sequences such as promoters, operators, repressors, enhancers, termination sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention. As used herein, a transcription control sequence includes a sequence which is capable of controlling the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, and mammalian cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, oxy-pro, omp/lpp, *rrnB*, bacteriophage lambda (λ) (such as λp_L and λp_R and fusions that include such promoters), bacteriophage T7, *T7lac*, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha mating factor, baculovirus, vaccinia virus, herpesvirus, poxvirus, adenovirus, simian virus 40, retrovirus actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences, as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins). Transcription control sequences of the present invention can also include naturally occurring transcription control sequences naturally associated with a DNA sequence encoding a MEKK protein.

Expression vectors of the present invention may also contain fusion sequences which lead to the expression of inserted nucleic acid molecules of the present invention as fusion proteins. Inclusion of a fusion sequence as part of a MEKK nucleic acid molecule of the present invention can enhance the stability during production, storage and/or use of the protein encoded by the nucleic acid molecule. Furthermore, a fusion segment can function as a tool to simplify purification of a MEKK protein, such as to enable purification of the resultant fusion protein using affinity chromatography. A suitable fusion segment can be a domain of any size that has the desired function (e.g., increased stability and/or purification tool). It is within the scope of the present invention to use one or more fusion segments. Fusion segments can be joined to amino and/or carboxyl termini of a MEKK protein. Linkages between fusion segments and MEKK proteins can be constructed to be susceptible to cleavage to enable straight-forward recovery of the MEKK proteins. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of a MEKK protein.

Expression constructs of the subject MEKK polypeptide, and mutants thereof, may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus,

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular MEKK polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding one of the subject proteins rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in *Current Protocols in Molecular Biology*, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψ Crip, ψ Cre, ψ 2

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julian et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *Biotechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al.

Yet another viral vector system useful for delivery of one of the subject MEKK genes is the adeno-associated virus (AMINO ACIDSV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AMINO ACIDSV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AMINO ACIDSV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AMINO ACIDSV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a subject MEKK polypeptide in the

In clinical settings, the gene delivery systems for the therapeutic MEKK gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A MEKK gene, such as any one of the clones represented in the appended Sequence Listing, can be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

Still another aspect of the present invention pertains to recombinant cells, e.g., cells which are transformed with at least one of any nucleic acid molecule of the present invention. A preferred recombinant cell is a cell transformed with at least one nucleic acid molecule that encodes at least a portion of a MEKK protein, or a homologue thereof. A more preferred recombinant cell is transformed with at least one nucleic acid molecule that is capable of encoding at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12 and/or SEQ ID No:14, or homologues thereof. An even more preferred recombinant cell is transformed with at least one nucleic acid molecule represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, and/or SEQ ID No:13, or complements thereof.

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant protein production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing the resultant protein.

As used herein, amplifying the copy number of a nucleic acid sequence in a cell can be accomplished either by increasing the copy number of the nucleic acid sequence in the cell's genome or by introducing additional copies of the nucleic acid sequence into the cell by transformation. Copy number amplification is conducted in a manner such that greater amounts of enzyme are produced, leading to enhanced conversion of substrate to product. For example, recombinant molecules containing nucleic acids of the present invention can be transformed into cells to enhance enzyme synthesis. Transformation can be accomplished using any process by which nucleic acid sequences are inserted into a cell. Prior to transformation, the nucleic acid sequence on the recombinant molecule can be manipulated to encode an enzyme having a higher specific activity.

In accordance with the present invention, recombinant cells can be used to produce a MEKK protein of the present invention by culturing such cells under conditions effective to produce such a protein, and recovering the protein. Effective conditions to produce a protein include, but are not limited to, appropriate media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An appropriate, or effective, medium refers to any medium in which a cell of the present invention, when cultured, is capable of producing a MEKK protein. Such a medium is typically an aqueous medium comprising assimilable carbohydrate, nitrogen and phosphate sources, as well as appropriate salts, minerals, metals

A preferred cell to culture is a recombinant cell that is capable of expressing the MEKK protein, the recombinant cell being produced by transforming a host cell with one or more nucleic acid molecules of the present invention. Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

With respect to methods for producing the subject MEKK polypeptide, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The cells may be harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant MEKK polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant MEKK polypeptide is a fusion protein containing a domain which facilitates its purification, such as GST fusion protein or poly(His) fusion protein.

Cells of the present invention can be cultured in conventional fermentation bioreactors, which include, but are not limited to, batch, fed-batch, cell recycle, and continuous fermentors. Culturing can also be conducted in shake flasks, test tubes, microtiter dishes, and petri plates. Culturing is carried out at a temperature, pH and oxygen content appropriate for the recombinant cell. Such culturing conditions are well within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant MEKK proteins may either remain within the recombinant cell or be secreted into the fermentation medium. The phrase "recovering the protein" refers simply to collecting the whole fermentation medium containing the protein and need not imply additional steps of separation or purification. MEKK proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction

Alternatively, a MEKK protein of the present invention can be produced by isolating the MEKK protein from cells or tissues recovered from an animal that normally express the MEKK protein. For example, a cell type, such as T cells, can be isolated from the thymus of an animal. MEKK protein can then be isolated from the isolated primary T cells using standard techniques described herein.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with upstream or downstream elements. Accordingly, in an exemplary screening assay of the present invention, the compound of interest is contacted with proteins which may function upstream (including both activators and repressors of its activity such as, Ras, Rac, Cdc 42 or Rho or other Ras superfamily members) or to proteins or nucleic acids which may function downstream of the MEKK polypeptide, whether they are positively or negatively regulated by it. For convenience, such polypeptides of a signal transduction pathway which interact directly with MEKK will be referred to below as MEKK-binding proteins (MEKK-bp). These proteins include the downstream targets of MEKKs, namely, members of the MAP kinase kinase family (MEKs or MKKs), as MEK1, MEK2, MKK1, MKK2, the stress-activated kinases (SEKs), also known as the Jun kinase kinases (JNKKs), MEKK3 and MEKK4 or the like. Other downstream targets of the MEKK family can include proteins from the mammalian MAP kinase family which includes, for example, the extracellular

inhibitory compound to inhibit phosphorylation of the MEKK substrate by the MEKK protein. A kit of the present invention can further comprise Raf protein and a means for detecting the ability of a putative inhibitory compound to inhibit the ability of Raf protein to phosphorylate the MEK protein.

In yet another embodiment, a mammalian MEKK gene can be used to rescue a yeast cell having a defective ste11 (or byr2) gene, such as a temperature sensitive mutant ste11 mutant (cf., Francois et al. (1991) *J Biol Chem* 266:6174-80; and Jenness et al. (1983) *Cell* 35:521-9). For example, a humanized yeast can be generated by amplifying the coding sequence of the human MEKK clone, and subcloning this sequence into a vector which contains a yeast promoter and termination sequences flanking the MEKK coding sequences. This plasmid can then be used to transform an ste11^{TS} mutant. To assay growth rates, cultures of the transformed cells can be grown at an permissive temperature for the TS mutant. Turbidity measurements, for example, can be used to easily determine the growth rate. At the non-permissive temperature, pheromone responsiveness of the yeast cells becomes dependent upon expression of the human MEKK protein. Accordingly, the humanized yeast cells can be utilized to identify compounds which inhibit the action of the human MEKK protein. It is also deemed to be within the scope of this invention that the humanized yeast cells of the present assay can be generated so as to comprise other human cell-cycle proteins. For example, human MEK and human MAPK can also be expressed in the yeast cell in place of ste7 and Fus3/Kss1. In this manner, the reagent cells of the present assay can be generated to more closely approximate the natural interactions which the mammalian MEKK protein might experience.

Furthermore, certain formats of the subject assays can be used to identify drugs which inhibit proliferation of yeast cells or other lower eukaryotes, but which have a substantially reduced effect on mammalian cells, thereby improving therapeutic index of the drug as an anti-mycotic agent. For instance, in one embodiment, the identification of such compounds is made possible by the use of differential screening assays which detect and compare drug-mediated disruption of binding between two or more different types of MEKK/MEKK-bp complexes, or which differentially inhibit the kinase activity of, for example, ste11 relative to a mammalian MEKK. Differential screening assays can be used to exploit the difference in drug-mediated disruption of human MEKK complexes and yeast ste11/byr2 complexes in order to identify agents which display a statistically significant increase in specificity for disrupting the yeast complexes (or kinase activity) relative to the human complexes. Thus, lead compounds which act specifically to inhibit proliferation of pathogens, such as fungus involved in mycotic infections, can be developed. By way of illustration, the present assays can be used to screen for agents which may ultimately be useful for inhibiting at least one fungus implicated in such mycosis as candidiasis, aspergillosis, mucormycosis,

to isolate genes that are turned on or turned off by this process. The powerful subtractive library methodology incorporating PCR technology described by Wang and Brown is an example of a methodology useful in conjunction with the present invention to isolate such genes (Wang et al. (1991) *PNAS* 88:11505-11509). Utilizing control and treated cells, the induced pool can be subtracted from the uninduced pool to isolate genes that are turned on, and then the uninduced pool from the induced pool for genes that are turned off. From this screen, it is expected that two classes of mRNAs can be identified. Class I RNAs would include those RNAs expressed in untreated cells and reduced or eliminated in induced cells, that is the down-regulated population of RNAs. Class II RNAs include RNAs that are upregulated in response to induction and thus more abundant in treated than in untreated cells. RNA extracted from treated vs untreated cells can be used as a primary test for the classification of the clones isolated from the libraries.

In still another embodiment of the present invention, compositions comprising MEKK therapeutics can be used for the *in vitro* generation of skeletal tissue, such as from skeletogenic stem cells, as well as for the *in vivo* treatment of skeletal tissue deficiencies. The present invention contemplates the use of MEKK therapeutics which upregulate or mimic the inductive activity of a bone morphogenetic protein (BMP) or TGF- β , such as may be useful to control chondrogenesis and/or osteogenesis. By "skeletal tissue deficiency", it is meant a deficiency in bone or other skeletal connective tissue at any site where it is desired to restore the bone or connective tissue, no matter how the deficiency originated, e.g. whether as a result of surgical intervention, removal of tumor, ulceration, implant, fracture, or other traumatic or degenerative conditions, so long as modulation of a TGF- β inductive response is appropriate.

For instance, the present invention makes available effective therapeutic methods and MEKK therapeutic compositions for restoring cartilage function to a connective tissue. Such methods are useful in, for example, the repair of defects or lesions in cartilage tissue which is the result of degenerative wear such as that which results in arthritis, as well as other mechanical derangements which may be caused by trauma to the tissue, such as a displacement of torn meniscus tissue, meniscectomy, a laxation of a joint by a torn ligament, malignment of joints, bone fracture, or by hereditary disease. The present reparative method is also useful for remodeling cartilage matrix, such as in plastic or reconstructive surgery, as well as periodontal surgery. The present method may also be applied to improving a previous reparative procedure, for example, following surgical repair of a meniscus, ligament, or cartilage. Furthermore, it may prevent the onset or exacerbation of degenerative disease if applied early enough after trauma.

The present invention further contemplates the use of the subject method in the field of cartilage transplantation and prosthetic device therapies. To date, the growth of new

allele which is mis-expressed. For example, a mouse can be bred which has one or more MEKK alleles deleted or otherwise rendered inactive. Such a mouse model can then be used to study disorders arising from mis-expressed MEKK genes, as well as for evaluating potential therapies for similar disorders.

Another aspect of the present invention concerns transgenic animals which are comprised of cells (of that animal) which contain a transgene of the present invention and which preferably (though optionally) express an exogenous MEKK protein in one or more cells in the animal. A MEKK transgene can encode the wild-type form of the protein, or can encode homologs thereof, including both agonists and antagonists, as well as antisense constructs. In preferred embodiments, the expression of the transgene is restricted to specific subsets of cells, tissues or developmental stages utilizing, for example, cis-acting sequences that control expression in the desired pattern. In the present invention, such mosaic expression of a MEKK protein can be essential for many forms of lineage analysis and can additionally provide a means to assess the effects of, for example, lack of MEKK expression which might grossly alter development in small patches of tissue within an otherwise normal embryo. Toward this end, tissue-specific regulatory sequences and conditional regulatory sequences can be used to control expression of the transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences.

Genetic techniques which allow for the expression of transgenes can be regulated via site-specific genetic manipulation *in vivo* are known to those skilled in the art. For instance, genetic systems are available which allow for the regulated expression of a recombinase that catalyzes the genetic recombination a target sequence. As used herein, the phrase "target sequence" refers to a nucleotide sequence that is genetically recombined by a recombinase. The target sequence is flanked by recombinase recognition sequences and is generally either excised or inverted in cells expressing recombinase activity. Recombinase catalyzed recombination events can be designed such that recombination of the target sequence results in either the activation or repression of expression of one of the subject MEKK proteins. For example, excision of a target sequence which interferes with the expression of a recombinant MEKK gene, such as one which encodes an antagonistic homolog or an antisense transcript, can be designed to activate expression of that gene. This interference with expression of the protein can result from a variety of mechanisms, such as spatial separation of the MEKK gene from the promoter element or an internal stop codon. Moreover, the transgene can be made wherein the coding sequence of the gene is flanked by recombinase recognition sequences and is initially transfected into cells in a 3' to 5' orientation with respect to the promoter element. In such an instance, inversion of the target sequence will reorient the

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For example, compounds such as protein kinase C or phospholipase C kinase, can provide the second "hit" needed to drive the divergent Ras-dependent pathway down the MEKK-dependent pathway in such a manner that JNK is preferentially activated over MAPK.

One embodiment of the present invention comprises a method for regulating the homeostasis of a cell comprising regulating the activity of a MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in the cell. As used herein, the term "homeostasis" refers to the tendency of a cell to maintain a normal state using intracellular systems such as signal transduction pathways. Regulation of the activity of a MEKK-dependent pathway includes increasing the activity of a MEKK-dependent pathway relative to the activity of a Raf-dependent pathway by regulating the activity of a member of a MEKK-dependent pathway, a member of a Raf-dependent pathway, and combinations thereof, to achieve desired regulation of phosphorylation along a given pathway, and thus effect apoptosis. Preferred regulated members of a MEKK-dependent pathway or a Raf-dependent pathway to regulate include, but are not limited to, proteins including MEKK, Ras, Rac, Cdc 42, Raf, MKK, JNKK, MEK, MAPK, JNK, TCF, ATF-2, Jun and Myc, and combinations thereof.

In one embodiment, the activity of a member of a MEKK-dependent pathway, a member of a Raf-dependent pathway, and combinations thereof, are regulated by altering the concentration of such members in a cell. One preferred regulation scheme involves altering the concentration of proteins including MEKK, Ras, Rac, Cdc 42, Raf, JNKK, MEK, MAPK, JNK, TCF, Jun, ATF-2, and Myc, and combinations thereof. A more preferred regulation scheme involves increasing the concentration of proteins including MEKK, Ras, Rac, Cdc 42, JNKK, JNK, Jun, ATF-2, and Myc, and combinations thereof. Another more preferred regulation scheme involves decreasing the concentration of proteins including Raf, MEK, MAPK, and TCF, and combinations thereof. It is also within the scope of the present invention that the regulation of protein concentrations in two or more of the foregoing regulation schemes can be combined to achieve an optimal apoptotic effect in a cell.

A preferred method for increasing the concentration of a protein in a regulation scheme of the present invention includes, but is not limited to, increasing the copy number of a nucleic acid sequence encoding such protein within a cell, improving the efficiency with which the nucleic acid sequence encoding such protein is transcribed within a cell, improving the efficiency with which a transcript is translated into such a protein, improving the efficiency of post-translational modification of such protein, contacting cells capable of producing such protein with anti-sense nucleic acid sequences, and combinations thereof.

In a preferred embodiment of the present invention, the homeostasis of a cell is controlled by regulating the apoptosis of a cell. A suitable method for regulating the apoptosis of a cell is to regulate the activity of a MEKK-dependent pathway in which the

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MEKK protein regulates the pathway substantially independent of Raf. A particularly preferred method for regulating the apoptosis of a cell comprises increasing the concentration of MEKK protein by contacting a cell with a nucleic acid molecule encoding a MEKK protein that possesses unregulated kinase activity. A preferred nucleic acid molecule with which to contact a cell includes a nucleic acid molecule encoding a MEKK protein represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID No:14, and combinations thereof. A more preferred nucleic acid molecule with which to contact a cell includes a nucleic acid molecule encoding a truncated MEKK protein having only the kinase catalytic domain (i.e., no regulatory domain) of a MEKK protein represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12 and/or SEQ ID No:14. An even more preferred nucleic acid molecule with which to contact a cell includes a nucleic acid molecule including the kinase catalytic domain of a MEKK protein, for example, MEKK1.1₄₀₉₋₆₇₂ MEKK1₁₃₂₉₋₁₅₉₄, MEKK2.1₃₆₁₋₆₂₀, MEKK2.2₃₆₁₋₆₂₀ MEKK3₃₆₆₋₆₂₆, MEKK4.1₆₃₁₋₈₉₀, MEKK4.2₁₃₃₈₋₁₅₉₇. Again, suitable variations of MEKK proteins described herein comprise those proteins encoded by a nucleic acid molecule that are able to hybridize to any of the above sequences under stringent conditions.

It is within the scope of the invention that the foregoing method can further comprise the step of decreasing the activity of MEK protein in the cell by contacting the cell with a compound capable of inhibiting MEK activity. Such compounds can include: peptides capable of binding to the kinase domain of MEK in such a manner that phosphorylation of MAPK protein by the MEK protein is inhibited; and/or peptides capable of binding to a portion of a MAPK protein in such a manner that phosphorylation of the MAPK protein is inhibited.

In another embodiment, the activity of a member of a MEKK-dependent pathway, a member of a Raf-dependent pathway, and combinations thereof, can be regulated by directly altering the activity of such members in a cell. A preferred method for altering the activity of a member of a MEKK-dependent pathway, includes, but is not limited to, contacting a cell with a compound capable of directly interacting with a protein including MEKK, Ras, Rac, Cdc 42, JNKK, JNK, Jun, ATF-2, and Myc, and combinations thereof, in such a manner that the proteins are activated; and/or contacting a cell with a compound capable of directly interacting with a protein including Raf, MEK, MAPK, TCF protein, and combinations thereof in such a manner that the activity of the proteins are inhibited. A preferred compound with which to contact a cell that is capable of regulating a member of a MEKK-dependent pathway includes a peptide capable of binding to the regulatory domain of proteins including MEKK, Ras, Rac, Cdc 42, JNKK, JNK, Jun, ATF-2, and Myc, in which the peptide inhibits the ability of the regulatory domain to regulate the activity of the kinase domains of such

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acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds as disclosed herein that are capable of inhibiting the binding of Ras superfamily protein to MEKK. A mimetope can also be obtained by, for example, rational drug design. In a rational drug design procedure, the three-dimensional structure of a compound of the present invention can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling. The predicted mimetope structures can then be produced by, for example, chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

In one embodiment, a Ras:MEKK binding compound of the present invention comprises an isolated peptide having a domain of a Ras superfamily protein that is capable of binding to a MEKK protein (i.e., that has an amino acid sequence which enables the peptide to be bound by a MEKK protein). A Ras peptide of the present invention is of a size that enables the peptide to be bound by a MEKK protein, preferably, at least about 4 amino acid residues, more preferably at least about 12 amino acid residues, and even more preferably at least about 25 amino acid residues. In particular, a Ras peptide of the present invention is capable of being bound by the COOH-terminal region of MEKK, in certain embodiments the region of MEKK containing the MEKK kinase domain. Preferably, a Ras peptide of the present invention comprises the effector domain of Ras and more preferably amino acid residues 17-42 of H-Ras. In addition, similar domains of Rac are important for the binding of Rac, Cdc 42 or Rho to certain MEKK proteins.

In another embodiment, a Ras:MEKK binding compound of the present invention comprises an isolated MEKK peptide that has a domain of a MEKK protein that is capable of binding to a Ras protein (i.e., that has an amino acid sequence which enables the peptide to be bound by a Ras protein). A MEKK peptide of the present invention is of a size that enables the peptide to be bound by a Ras protein, in particular by the effector domain of a Ras protein. Preferably, a MEKK peptide of the present invention at least about 320 amino acids in length. Preferably, a MEKK peptide of the present invention comprises the COOH-terminal region of a MEKK protein and more preferably MEKK_{COOH} (as described in detail in the appended examples).

As an illustrative example, the sequence of a MEKK protein which binds to Cdc42 and Rac, such as IIGQVCDTPKSYDNVMHVGLR, occurring around residue 1306-1326 of MEKK4.2 or 599-619 of MEKK4 or mimetics thereof could be used therapeutically. In one embodiment the Rac-binding portion of a MEKK protein or a fragment thereof is used to

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Ras is a critical component of tyrosine kinase growth factor receptor and G-protein coupled receptor regulation of signal transduction pathways controlling mitogenesis and differentiation. According to the present invention, the protein serine-threonine kinases Raf-1 and MEKK1 are Ras effectors and selectively bind to Ras in a GTP dependent manner. The p110 catalytic subunit of the lipid kinase has also been shown to directly interact with Ras in a GTP dependent manner. Ras-GAP and neurofibromin also regulate Ras GTPase activity. Raf-1, MEKK1 and PI3-kinase are capable of increasing the activity in cells expressing GTPase-deficient Ras consistent with their interaction with Ras-GTP being involved in their regulation.

As additional MEKKs are characterized it will be important to characterize their regulation and interaction with other members of the Ras superfamily. For example, MEKK4.1 and 4.2 have been found to bind to Rac/Cdc42 as described herein. Rho, Rac, and Cdc42 are small GTPases that have been implicated in the formation of a variety of actin structures and the assembly of associated integrin complexes (Burbelo, et al. (1995) J. Biol

Compounds of the present invention may influence cellular mitogenesis, DNA synthesis, cell division and differentiation. MAPK is also recognized as being involved in the activation of oncogenes, such as *c-jun* and *c-myc*. While not bound by theory, the present inventor believes that MAPK is also intimately involved in various abnormalities having a genetic origin. MAPK is known to cross the nuclear membrane and is believed to be at least partially responsible for regulating the expression of various genes. As such, MAPK is believed to play a significant role in the instigation or progression of cancer, neuronal diseases, autoimmune diseases, allergic reactions, wound healing and inflammatory responses. The present inventor, by being first to identify nucleic acid sequences encoding MEKK, recognized that it is now possible to regulate the expression of MEKK, and thus regulate the activation of MAPK.

A naked plasmid DNA compound of the present invention is capable of treating a subject suffering from a medical disorder including cancer, autoimmune disease,

An isolated compound of the present invention can be used to formulate a therapeutic composition. In one embodiment, a therapeutic composition of the present invention includes at least one isolated peptide of the present invention. A therapeutic composition for use with a treatment method of the present invention can further comprise suitable excipients. A therapeutic compound for use with a treatment method of the present invention can be formulated in an excipient that the subject to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful excipients include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal, m- or o-cresol, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable

In another embodiment, a therapeutic compound for use with a treatment method of the present invention can also comprise a carrier. Carriers are typically compounds that increase the half-life of a therapeutic compound in the treated animal. Suitable carriers include, but are not limited to, liposomes, micelles, cells, polymeric controlled release formulations, biodegradable implants, bacteria, viruses, oils, esters, and glycols. Preferred carriers include liposomes and micelles.

A therapeutic compound for use with a treatment method of the present invention can be administered to any subject having a medical disorder as herein described. Acceptable protocols by which to administer therapeutic compounds of the present invention in an effective manner can vary according to individual dose size, number of doses, frequency of dose administration, and mode of administration. Determination of such protocols can be accomplished by those skilled in the art without resorting to undue experimentation. An effective dose refers to a dose capable of treating a subject for a medical disorder as described herein. Effective doses can vary depending upon, for example, the therapeutic compound used, the medical disorder being treated, and the size and type of the recipient animal. Effective doses to treat a subject include doses administered over time that are capable of regulating the activity, including growth, of cells involved in a medical disorder. For example, a first dose of a naked plasmid DNA compound of the present invention can comprise an amount that causes a tumor to decrease in size by about 10% over 7 days when administered to a subject having a tumor. A second dose can comprise at least the same the same therapeutic compound than the first dose.

Another aspect of the present invention includes a method for prescribing treatment for subjects having a medical disorder as described herein. A preferred method for prescribing treatment comprises: (a) measuring the MEKK protein activity in a cell involved in the medical disorder to determine if the cell is susceptible to treatment using a method of the present invention; and (b) prescribing treatment comprising regulating the activity of a MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in the cell to induce the apoptosis of the cell. The step of measuring MEKK protein activity can comprise: (1) removing a sample of cells from a subject; (2) stimulating the cells with a $\text{TNF}\alpha$; and (3) detecting the state of phosphorylation of MKK3, MKK4 or JNKK protein using an immunoassay using antibodies specific for phosphothreonine and/or phosphoserine.

The present invention also includes antibodies capable of selectively binding to a MEKK protein of the present invention. Such an antibody is herein referred to as an anti-MEKK antibody. Polyclonal populations of anti-MEKK antibodies can be contained in a

Antibodies of the present invention can be either polyclonal or monoclonal antibodies and can be prepared using techniques standard in the art. Antibodies of the present invention include functional equivalents such as antibody fragments and genetically-engineered antibodies, including single chain antibodies, that are capable of selectively binding to at least one of the epitopes of the protein used to obtain the antibodies. Preferably, antibodies are raised in response to proteins that are encoded, at least in part, by a MEKK nucleic acid molecule. More preferably antibodies are raised in response to at least a portion of a MEKK protein, and even more preferably antibodies are raised in response to either the amino terminus or the carboxyl terminus of a MEKK protein. Preferably, an antibody of the present invention has a single site binding affinity of from about $10^{-3}M$ to about $10^{-12}M$ for a MEKK protein of the present invention.

Another aspect of the present invention comprises a therapeutic compound capable of regulating the activity of a MEKK-dependent pathway in a cell identified by a process, comprising: (a) contacting a cell with a putative regulatory molecule; and (b) determining the ability of the putative regulatory compound to regulate the activity of a MEKK-dependent pathway in the cell by measuring the activation of at least one member of said MEKK-dependent pathway. Preferred methods to measure the activation of a member of a MEKK-dependent pathway include measuring the transcription regulation activity of c-Myc protein, measuring the phosphorylation of a protein selected from the group consisting of MEKK, JNKK, JNK, Jun, ATF-2, Myc, and combinations thereof.

Mitogen-activated protein kinase kinase (MEKK1) is a serine/threonine protein kinase that functions parallel to Raf-1 in the regulation of sequential protein kinase pathways that involve both mitogen-activated and stress-activated protein kinases. In this study, we examined the interaction of MEKK1 with 14-3-3 proteins. The T cell 14-3-3 isoform, but not

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

Example 1. This example describes the structural characterization of MEKK1 protein.

MEKK1.1 and 1.2 protein was cloned by the following method. Unique degenerate inosine oligodeoxynucleotides were designed to correspond to regions of sequence identity between the yeast *Ste11* and *Byr2* genes. With primers and cDNA templates derived from polyadenylated RNA from NIH 3T3 cells, a polymerase chain reaction (PCR) amplification product of 320 base pairs (bp) was isolated. This 320 bp cDNA was used as a probe to identify a MEKK1.2 cDNA of 3260 bp from a mouse brain cDNA library using standard methods in the art. The MEKK1.2 nucleotide sequence was determined by dideoxynucleotide sequencing of double-stranded DNA using standard methods in the art.

Referring to SEQ ID No. ~~7~~, based on the Kozak consensus sequence for initiation codons, the starting methionine can be predicted to occur at nucleotide 486. With this

methionine at the start, the cDNA encodes a protein of 672 amino acids, corresponding to a molecular size of 73 kD. When run on a gel, the protein has an apparent molecular size of 69 kD. There is another in-frame methionine at position 441, which does not follow the Kozak rule, but would yield a protein of 687 amino acid residues (74.6 kD). Also referring to SEQ ID No:2, 20% of the NH₂-terminal 400 amino acids are serine or threonine and there are only two tyrosines. Several potential sites of phosphorylation by protein kinase C are apparent in the NH₂-terminal region. The kinase catalytic domain is located in the COOH-terminal half of the MEKK 1.

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B. ^{Northern} ~~Southern~~ Blot Analysis of MEKK 1 Transcript

Equal amounts (20 µg) of total RNA were loaded onto the gel as indicated by ethidium bromide staining. Blots were probed with either a 320-bp cDNA fragment encoding a portion of the MEKK kinase domain or an 858-bp fragment encoding a portion of the NH₂ terminal region of MEKK using standard methods in the art. A 7.8 kb mRNA was identified with probes derived from both the 5' and 3' ends of the MEKK cDNA in several cell lines and mouse tissues. The MEKK mRNA was highly expressed in mouse heart and spleen, an in lower amounts in liver.

C. Southern Blot Analysis

Mouse genomic DNA (10 µg) was digested with either *Bam* HI, *Hind* III or *Eco* RI and applied to gels using standard methods in the art. Blots were probed with a 320-bp fragment of the *MEKK* gene. The appearance of one band was detected in the *Bam* HI and *Hind* III digests which indicates that MEKK is encoded by one gene. The appearance of two bands in the *Eco* RI digest indicates the likely presence of an *Eco* RI site within an intron sequence spanned by the probe.

D. Immunoblots Using Anti-MEKK Antibodies

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Three polyclonal antisera were prepared using three different antigens. A first polyclonal antiserum was prepared using an antigen comprising a 15 amino acid peptide (corresponding to amino acids 655 to 669 of SEQ ID No:2) DRPPSRELLKHPVER_N derived from the COOH-terminus of MEKK. NZW rabbits were immunized with the peptide and antisera was recovered using standard methods known in the art. This first polyclonal antiserum is hereinafter referred to as the DRPP antiserum.

A second polyclonal antiserum was produced using a DNA clone comprising a MEKK cDNA digested with *Eco*R1 and *Pst*I, thereby creating a 1270 bp fragment that encodes the amino terminus of MEKK. This fragment was cloned into pRSETC to form the recombinant molecule pMEKK1-369 comprising amino acid residues 1 to 369 of MEKK1. The pMEKK11-369 recombinant molecule was expressed in *E. coli* and protein encoded by the recombinant molecule was recovered and purified using standard methods known in the art. NZW rabbits were immunized with the purified recombinant MEKK11-369 protein and

Using the same procedure described above, two MEKK immunoreactive species of approximately 98 kD and 82 kD present in PC12, Rat1a, NIH3T3, and Swiss3T3 cell lysates were recognized by affinity purified MEKK11-369 antiserum. It should be noted that the 98 kD MEKK protein described herein was originally identified as a 95 kD MEKK protein in the related PCT application (International application no. PCT/US94/04178). Subsequent

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Cloning of MEKK 2 and 3.

SEQ ID NO: 15

The degenerate primers GA(A/G)(C/T)TATGGCIGTI

AA(A/G)CA₁(sense) and TTIGCICCC(T/C)TTIAT(A/G)TCIC(G/T)(A/G)TG₁(antisense) were used in a PCR using first strand cDNA generated from polyadenylated RNA prepared from NIH 3T3 cells. The PCR reaction involved 30 cycles (1 min, 94°C; 2 min, 52°C; 3 min 72°C). A band of approximately 300 base pairs was recovered from the PCR mixture, and the products were cloned into pGEM-T (Promega). The PCR cDNA products were sequenced and compared to the MEKK1 sequence. A unique cDNA sequence of 322 base pairs having significant homology to MEKK1 cDNA was identified and used to screen an oligo (dT)-primed mouse brain cDNA library (Stratagene). The λ phage library was plated and DNA from plaques was transferred to Hybond N filters (Amersham) followed by UV-cross-linking of DNA to the filters. Filters were prehybridized for 2 h and then hybridized overnight in 0.5M Na₂H₂PO₄ (pH 7.2), 10% bovine serum albumin, 1 mM EDTA, 7% SDS at 68°C. Filters were washed twice at 42°C with 2 x SSC, once with 1X SSC, and once with 0.5x SSC containing 0.1% SDS. Positive hybridizing clones were purified and sequenced. To resolve GC-rich regions, cDNAs were subcloned into M13 vectors (New England Biolabs), and single strand DNA was sequenced. In all cases, both strands of DNA were sequenced. MEKK 2 encodes a 619-amino acid protein having a mass of 69.7 kDa. MEKK 3 encodes a 626-amino acid protein having a mass of 71 kDa. The two proteins share a common structure with the kinase catalytic domain encoded in the COOH-terminal moiety. The amino-terminal moiety does not encode any definable domain such as a SH2 or SH3 domain sequence.

The 5' ends of both MEKK 2 and 3 are highly G/C-rich making DNA sequencing difficult. To verify the presence of stop codons in all three possible reading frames 5' to the predicted start site methionine, the MEKK 2 and 3 cDNAs were inserted in pRSET A, B, and

C (Invitrogen) and expressed in *Escherichia coli*. Each construct gave a truncated RSET fragment confirming that the MEKK 2 and 3 cDNAs encoded 5' stop sites and that the isolated cDNAs encode full-length proteins.

Alignment of the deduced amino acid sequences demonstrated significant homology between the two proteins. Overall, the two proteins are approximately 77% homologous. The catalytic domain is encoded in the COOH-terminal moiety of both MEKK 2 and 3. The first consensus kinase domain comprising the catalytic site of MEKK 2 and 3 begins at residues 361 and 367, respectively. The COOH-terminal catalytic domains of MEKK 2 and 3 are approximately 94% conserved, whereas the NH₂-terminal moieties are only 65% conserved in amino acid sequence. These findings indicate that the primary sequences of MEKK 2 and 3 diverge significantly in the NH₂-terminal half of the proteins. The conservation in sequence of the catalytic domains suggests they may recognize an overlapping set of substrates. The divergent NH₂ termini would be consistent with this region encoding sequences for the differential regulation of the two proteins.

The COOH terminus of MEKK 1 encoding the catalytic domain is only 50% homologous to the corresponding regions of MEKK 2 and 3. Thus, the catalytic domains of MEKK 2 and 3 are very similar to each other but significantly divergent from MEKK 1. As shown below, MEKK 1, 2, and 3 can all stimulate JNK and p42/44^{MAPK} activities in transfected cells. The significance of the sequence differences in the catalytic domains of MEKK 1, 2, and 3 is presently unclear.

Plasmid Expression of MEKK2 and 3. The proteins for MEKK2 and 3 were epitope-tagged at their NH₂ terminus with the hemagglutinin (HA) tag sequence GYPYDVPDYA^{SEQ ID NO:17} using a PCR strategy. For inserting the NH₂-terminal epitope tag in MEKK2 and 3, sense oligonucleotides were synthesized having a methionine codon (ATG), 33 bases coding for the GYPYDVPDYA^{SEQ ID NO:17} epitope tag sequences, and 20 bases of MEKK 2 or 3 sequence starting at codon 2. For MEKK2, the sense oligonucleotide was ATGGGGTACCCGTACGACGTGCCGGACTACGCTTCCGATGATCAGCAAGCTTTGA^{SEQ ID NO:18} the sense oligonucleotide for MEKK3 was ATGGGGTACCCGTACGACGTGCCGGACTACGCTTCCGATGAACAAGAGGCATTAGA^{SEQ ID NO:19} The antisense oligonucleotides for MEKK2 and 3 were AGACTTAGATCTCAGGTCTTC^{SEQ ID NO:20} encoding a BglII site for MEKK2 and GATTCTGACGTCCTCTGCCT^{SEQ ID NO:21} encoding an ActII site for MEKK3. The PCR reactions were performed for 30 cycles using MEKK2 or MEKK3 cDNAs as template. The PCR products were purified, and a second PCR reaction was performed using the first PCR product as template, the MEKK2 or 3 antisense oligonucleotide described above and the common sense oligonucleotide encoding a XbaI restriction site, a consensus Kozak initiation site and 17 bases overlapping with the initiation methionine and HA tag sequence

Transient expression of MEKK 2 and 3 also stimulated p42/44^{MAPK} activity. Immunoblotting of hemagglutinin (HA) epitope-tagged MEKK 2 and 3 indicated that MEKK 2 and 3 were expressed at similar levels in HEK293 cells when 2 pg of plasmid DNA was used per transfection. MAPK activity following Mono Q FPLC fractionation was measured using the epidermal growth factor receptor 662-631 peptide as a selective p42/44 MAPK substrate. Alternatively, for cells transfected with varying amounts of MEKK plasmids,

MEKK 2 Phosphorylates Both MEK 1 and JNK Kinase in Vitro. HEK293 cells expressing MEKK 2 and 3 were lysed in 1% Triton x-100, 0.5% Nonidet P-40, 20 mM Tris HCL, pH 7.5, 150 mM NaCl, 20 mM NaF, 0.2 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 5 mM PMSF. Nuclei were removed by centrifugation at 15,000 x g for 5 min. HA epitope-tagged MEKK2 and 3 were immunoprecipitated with the 12CA5 antibody recognizing the HA epitope-tag. The immunoprecipitates were washed twice in lysis buffer, twice in PAN (10 mM Pipes, pH 7.0, 100 mM NaCl, 20 ug/ml aprotinin), suspended in 20 mM Pipes, 10 mM MnCl₂, 20 ug/ml aprotinin, and used in an in vitro kinase assay with 20-50 ng of recombinant MEK1 or JNKK as substrates and 20 uCi of [γ -³²P]ATP. Reactions were terminated by the addition of Laemmli sample buffer, boiled, and proteins were resolved by SDS-10% PAGE.

MEKK 2 clearly phosphorylates both MEK 1 and JNKK consistent with its ability to activate JNK and p42/44^{MAPK} in HEK298 cells. MEKK 2-catalyzed phosphorylation of recombinant JNKK resulted in the enhancement of JNKK activity. Thus, JNKK is a MEKK 2 substrate whose activity is stimulated both *in vitro* and *in vivo* by MEKK 2. We were unable to demonstrate the ability of MEKK 3 to phosphorylate MEK 1, MEK 2, or JNKK *in vitro* using a variety of immunoprecipitation procedures. Although MEKK 3 was efficiently immunoprecipitated, as determined by Western blot analysis, it did not show measurable kinase activity toward MEK 1 or JNKK or show detectable autophosphorylation. This

MEKK 2 and 3 Do Not Regulate p38 Activity in HEK293 Cells. The p38 kinase is activated by hypersmotic conditions and recognizes the transcription factor ATF 2 as an *in vitro* substrate. Sorbitol treated (0.4M, 20 min) or control HEK293 cells were lysed in the same buffer as that used for immunoprecipitation of p38 using rabbit antiserum raised against the COOH terminal peptide sequence of p38. Immunoprecipitates were washed once in lysis buffer, once in assay buffer (25 mM Hepes, pH 7.4, 25 mM β -glycerophosphate, 25 mM NaCl₂, 2 mM dithiothreitol, 0.1 mM sodium vanadate) resuspended, and used in an invitro kinase assay with a recombinant NH₂-terminal fragment of ATF 2 (20-50 ng). For analysis of p38 kinase activity from Mono Q FPLC fractions, 20 ul aliquots were mixed with kinase buffer containing 20-50 ng of recombinant ATF 2 and 10 uCi of [γ 32P]PATP. Reactions were quenched in Laemmli sample buffer, boiled, and proteins were resolved using SDS 10% PAGE. Immunoprecipitation and *in vitro* kinase assay of p38 from MEKK 2 and 3 transfected HEK293 cells indicated that neither MEKK 2 nor MEKK 3 stimulated p38 kinase activity. Mono Q FPLC fractionation of lysates from MEKK 2 or 3 transfected HEK293 cells confirmed that p38 kinase activity was similar to that from control transfected cells. ATF 2 is also a substrate for JNK. Fractions 2-8 from cells transfected with MEKK 2 or 3, that contain immunoreactive JNK, have increased kinase activity toward ATF 2. This is a predicted result based on the ability of both MEKK 2 and 3 to stimulate JNK activity in HEK293 cells. Expression of MEKK 2 and 3 also activated additional ATF 2 phosphorylating activities resolved by Mono Q fractionation. These activities are seen to elute in fractions 9-12 and 13-18 for lysates from both MEKK 2 and 3 expressing cells. These activities do not correspond by immunoblotting to JNK, p42/44^{MAPK}, p88, or MEKK

Example 3. This example describes the expression of MEKK 1 protein in COS-1 cells to define its function in regulating the signaling system that includes MAPK.

COS cells in 100-mm culture dishes were transfected with either the pCVMV5 expression vector alone (1 μ g: control) or the pCVMV5 MEKK construct (1 μ g: MEKK). After 48 hours, the cells were placed in serum-free medium containing bovine serum albumin (0.1 percent) for 16 to 18 hours to induce quiescence. Cells were then treated with human EGF (30 ng/ml)(+EGF) or buffer (control) for 10 minutes, washed twice in cold phosphate buffered saline (PBS), and lysed in cell lysis buffer containing 50 mM β -glycerophosphate (pH 7.2), 100 μ M sodium vanadate, 2 mM $MgCl_2$, 1mM EGTA Triton X-100 (0.5 percent), leupeptin (2 μ g/ml), aprotinin (2 μ g/ml), and 1 mM dithiothreitol (600 μ l). After centrifugation for 10 minutes at maximum speed in a microfuge, COS cell lysates containing 0.5 to 1 mg of soluble protein were subjected to FPLC on a MONO Q column, and eluted fractions were assayed for MAPK activity according to the method described in Heasley et al., p. 545, 1992, *Mol. Biol. Cell*, Vol. 3.

Referring to Figure 3, when MEKK 1 was overexpressed in COS 1 cells, MAPK activity was four to five times greater than that in control cells transfected with plasmid lacking a MEKK 1 cDNA insert. The activation of MAPK occurred in COS cells deprived of serum and in the absence of any added growth factor. The activity of MAPK was similar to that observed after stimulation of control cells with EGF. Stimulation of COS cells transiently overexpressing MEKK with EGF resulted in only a slight increase in MAPK activity compared to that observed with MEKK expression alone.

To ensure that MEKK protein was present in the samples tested for MAPK activity, protein from cell lysates of the transfected COS 1 cells were immunoblotted with MEKK specific antiserum. Equal amounts (100 μ g) of soluble protein lysate from COS cells were placed on the gel for immunoblotting using the methods described in Example 1. The filters were immunoblotted using the affinity purified DRPP antiserum (1:300) and affinity purified MEKK1-369 antiserum (1:300). The results indicate that expression of MEKK in cells transfected with vector encoding MEKK that were treated with or without EGF. Only the 50 kD MEKK immunoreactive fragment was detected in lysates from control COS cells using the DRPP antiserum. Transient expression of MEKK in COS cells yielded a predominant 82 kD band that was slightly larger than that observed in PC12, Rat 1a, or NIH 3T3 cells. Addition of the 15 amino acid DRPP peptide antigen to the antiserum during immunoblotting prevented detection of all of the immunoreactive bands; these bands were not detected in

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SEQ ID NO: 25

Recombinant MAPK was used to assay MEK activity in COS cell lysates that had been fractionated by fast protein liquid chromatography (FPLC) on a Mono S column. A cDNA encoding p42 MAPK from *Xenopus laevis* was cloned into the pRSETB expression vector. This construct was used for expression in the LysS strain of *Escherichia coli* BL21(DE3) of a p42 MAPK fusion protein containing a polyhistidine sequence at the NH₂-terminus. Cultures containing the expression plasmid were grown at 37°C to an optical density of 0.7 to 0.9 at 600 nm. Isopropyl-β-thiogalactopyranoside (0.5 mM) was added to induce fusion protein synthesis and the cultures were incubated for 3 hours. The cells were then collected and lysed by freezing, thawing, and sonication. The lysate was centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was then passed over a Ni²⁺-charged Sepharose resin and the soluble recombinant MAPK was eluted in sodium phosphate buffer (pH 4.5). The purified recombinant MAPK was more than 80 percent pure. The purified recombinant MAPK served as a substrate for MEK and catalyzed the phosphorylation of a peptide consisting of residues 662 to 681 of the EGF receptor (EGFR⁶⁶²⁻⁶⁸¹).

Soluble cell lysates from COS cells transiently transfected with MEKK, mock-transfected (control), or mock-transfected and treated with EGF (30 ng/ml) (+EGF), were fractionated by FPLC on a Mono S column and endogenous MEK activity was measured. Endogenous MAPK eluted in fractions 2 to 4, whereas MEK was contained in fractions 9 to 13. For assaying endogenous MEK activity, cells were washed twice in cold PBS and lysed in 650 μ l of a solution containing 50 mM β -glycerophosphate, 10 mM 2-*N*-morpholinoethanesulfonic acid (pH 6.0), 100 μ M sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, Triton X-100 (0.5 percent), leupeptin (5 μ g/ml), aprotinin (2 μ g/ml), and 1 mM dithiothreitol. After centrifugation at maximum speed for 10 minutes in a microfuge, soluble cell lysates (1 to 2 mg of protein) were applied to a Mono S column equilibrated in elution buffer (50 mM β -glycerophosphate, 10 mM MES (pH 6.0), 100 μ M sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM dithiothreitol). The column was washed with buffer (2 ml) and bound proteins were eluted with a 30ml linear gradient of 0 to 350 mM NaCl in elution buffer. A portion (30 μ l) of each fraction was assayed for MEK activity by mixing with buffer (25 mM β -glycerophosphate, 40 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanolsulfonic acid) (pH 7.2) 50 mM sodium vanadate, 10 mM $MgCl_2$, 100 μ M γ - ^{32}P -ATP (3000 to 4000 cpm/pmol), inhibitor protein-20 (IP-20; TTYADFIASGRTGRRNAIHD; 25 μ g/ml), 0.5 mM EGTA, recombinant MAP kinase (7.5 μ g/ml), and 200 μ M EGFR⁶⁶²⁻⁶⁸¹) in a final volume of 40 μ l.

Phosphorylation of catalytically inactive MEK-1 by MEKK was time dependent; MEKK was also phosphorylated. Fraction 22 from FPLC on a Mono Q column (20 μ l) was incubated with or without recombinant catalytically inactive MEK-1 (0.15 μ g) for the indicated times. Phosphorylation of kinase MEK-1 and MEKK was visible after 5 minutes and maximal after about 20 minutes. The time-dependent increase in MEKK phosphorylation correlated with a decreased mobility of the MEKK protein during SDS-PAGE. Immunoblotting demonstrated that the MEKK protein co-eluted (after FPLC on a Mono Q column) with the peak of activity (fraction 22) that phosphorylated MEK. The slowly migrating species of MEKK were also detected by immunoblotting. Thus, expression of MEKK appears to activate MAPK by activating MEK.

Example 6. This Example describes that the phosphorylation of MEK by overexpressed MEKK resulted in activation of MEK, recombinant wild-type MEK-1 and a modified form of MAPK that is catalytically inactive.

COS cell lysates were separated by Mono Q-FPLC and fractions containing MEKK were assayed for their ability to activate added wild-type MEK-1 such that it would phosphorylate catalytically inactive recombinant MAPK in the presence of γ - 32 P-ATP. Lysates from COS cells transfected with MEKK or mock-transfected (control) were fractionated by FPLC on a Mono Q column and portions (20 μ l) of fractions containing MEKK were mixed with buffer. Each fraction was incubated in the presence (+) or absence (-) of purified recombinant wild-type MEK-1 (150 ng) and in the presence of purified recombinant, catalytically inactive (kinase-) MAPK (300 ng). Fractions 20 to 24 from lysates of COS cells transfected with MEKK activated MEK-1. Thus, MEKK phosphorylated and activated MEK-1, leading to MAPK phosphorylation.

Example 7. This Example describes studies demonstrating that MEKK activated MEK directly, and not through the activation of one or more other kinases contained in the column fractions.

Overexpressed MEKK was immunoprecipitated from COS cell lysates with affinity purified MEKK1-369 antiserum. Immunoprecipitated MEKK was resuspended in 10 to 15 μ l of PAN (10 mM piperazine-N, N'-bis-2-ethanesulfonic acid (Pipes) (pH 7.0), 100 mM NaCl, and aprotinin (20 μ g/ml) and incubated with (+) or without (-) catalytically inactive MEK-1 (150 ng) and 25 μ Ci of γ - 32 P-ATP in 20 mM pipes (pH 7.0), 10 mM MnCl_2 , and aprotinin (20 μ g/ml) in a final volume of 20 μ l for 15 minutes 30°C. Reactions were stopped by the addition of 5 x SDS sample buffer (5 μ l). The samples were boiled for 3 minutes and subjected to SDS-PAGE and autoradiography.

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Cell lysates prepared from EGF-stimulated and unstimulated cells were fractionated by FPLC on a Mono-Q column to partially purify the endogenous MEKK. Lysates from unstimulated control PC12 cells or cells treated with EGF (30ng/ml) for 5 minutes were fractionated by FPLC on a Mono Q column using a linear gradient of 0 to 525 mM NaCl. A portion (30 μ l) of each even numbered fraction was mixed with buffer (20mM piperazine-N,N'-bis-2-ethanesulfonic acid (Pipes) (pH 7.0), 10mM MnCl₂, aprotinin (20 μ g/ml), 50mM β -glycerophosphate (pH 7.2), 1mM EGTA, IP-20 (50 μ g/ml), 50mM NaF, and 30 μ Ci (γ -³²P)ATP) containing purified recombinant MEK-1 (150ng) as a substrate in a final volume of 40 μ l and incubated at 30°C for 25 minutes. Reactions were stopped by the addition of 2X SDS sample buffer (40 μ l), boiled and subjected to SDS-PAGE and autoradiography. The peak of MEKK activity eluted in fractions 10-12. Portions (30 μ l) of each even numbered fraction from lysates of EGF-treated PC12 cells were mixed with buffer as described above except containing purified recombinant MEKK NH₂-terminal fusion protein (400ng) as a substrate instead of MEK-1. Purified recombinant kinase inactive MEK-1 or the MEKK NH₂-terminal fusion protein were then used as substrates in the presence of (γ -³²P)ATP to determine whether 98 kD MEKK directly phosphorylates either substrate. Fractions 10-14 of lysate from PC12 cells treated with EGF phosphorylated MEK-1 while little MEK-1 phosphorylation occurred in untreated control fractions. The MEKK NH₂-terminal fusion protein was also phosphorylated in the same fractions as was MEK-1, although the peak of activity was slightly broader (fractions 8-16).

Immunoblotting of column fractions demonstrated that the 98 kD MEKK protein co-eluted with the peak of activity that phosphorylated either exogenously added kinase inactive MEK-1 or the 50 kD MEKK NH₂-terminal fusion protein. Portions (900 μ l) of even numbered column fractions were concentrated by precipitation with trichloroacetic acid and immunoblotted with MEKK antibody. The peak of immunoreactivity eluted in fractions 10-12.

Example 12. This Example describes the activation of MEK by a 98 kD MEKK.

98 kD MEKK was immunoprecipitated using the MEKK₁₋₃₆₉ antiserum described in Example 1 from untreated (-) or EGF-treated (+) PC12 cell lysates. The immunoprecipitates were incubated in the presence (+) or absence (-) of purified recombinant wild-type MEK (150 ng) and in the presence of purified recombinant catalytically inactive MAPK (300 ng) and (γ -³²P)ATP. The results indicate that immunoprecipitated MEKK from EGF-stimulated cells phosphorylated and activated MEK, leading to MAPK phosphorylation. No phosphorylation of MAPK occurred in the absence of added recombinant MEK. Immunoblotting demonstrated that there was no contaminating MAPK or contaminating MEK in the MEKK immunoprecipitates from the EGF-stimulated PC12 cells. Thus,

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Example 13. This Example describes whether 98 kD PC12 cell MEKK and Raf-B require functional Ras proteins for growth factor mediated signaling.

Dominant negative Ha-ras(Asn 17) (N¹⁷Ras) was expressed in PC12 cells and EGF-stimulated MEKK or Raf-B activation was assayed in immunoprecipitates using kinase inactive MEK-1 as a substrate. PC12 cells stably expressing dexamethasone inducible N¹⁷Ras were serum starved for 18-20 hours in media containing 0.1% BSA with or without 1 μ M dexamethasone and then untreated or treated with EGF (30ng/ml) for 5 minutes. Equal amounts of soluble protein from cell lysates was immunoprecipitated with either MEKK or Raf-B antisera and incubated with purified recombinant catalytically inactive MEK-1 and (γ -³²P)ATP as described above. Expression of N¹⁷Ras was induced in PC12 clones stably transfected with the N¹⁷Ras gene by the addition of dexamethasone to the starvation media. As shown in Figure 9, N¹⁷Ras expression inhibited the activation of MEKK by EGF as measured by its ability to phosphorylate kinase inactive MEK. EGF-mediated activation of Raf-B was also greatly reduced in N¹⁷Ras expressing PC12 cells compared to uninduced N¹⁷Ras transfectants. Addition of dexamethasone to wild type PC12 cells had no effect on the magnitude of MEKK or Raf-B activation elicited by EGF. PC12 cell clones stably transfected with the N¹⁷Ras gene are less responsive to EGF-mediated activation of MEKK activity than are wild type PC12 cells. These results indicate that functional Ras is required for growth factor stimulated activation of both Raf-B and MEKK in PC12 cells, suggesting that Ras may mediate its effects on cell growth and differentiation through the activation of multiple protein kinase effectors from both the Raf and MEKK families. Thus, EGF stimulated a peak of MEKK activity within 5 minutes which persisted for at least 30 minutes following treatment, and was similar to the timecourse of Raf-B activation. Nerve growth factor (NGF) and the phorbol ester TPA also activated MEKK, although to a lesser degree than EGF. MEKK activity in immunoprecipitates or column fractions was dissociable from that of EGF-stimulated c-Raf-1 and Raf-B activities. Forskolin pretreatment abolished both MEKK and Raf-B activation by EGF, NGF, and TPA (Figure 10). Both MEKK and Raf-B activation in response to EGF was inhibited by stable expression of dominant negative N¹⁷Ras. These findings represent the first demonstration of Ras-dependent MEKK regulation by growth factors and suggest the emergence of a complex intracellular kinase network in which Ras may alternately couple between members of the Raf and MEKK families.

To determine whether the growth factor-mediated activation of 98 kD PC12 cell MEKK was inhibited by PKA, forskolin was used to elevate intracellular cAMP and activate PKA. Serum-starved PC12 cells were pretreated with or without forskolin (50 μ M) for 3

minutes to activate protein kinase A and then with EGF (30ng/ml), NGF (100ng/ml), or TPA (200nM) for 5 minutes and MEKK was immunoprecipitated from equal amounts of soluble protein from cell lysates and incubated with purified recombinant catalytically inactive MEK-1 and (γ - 32 P)ATP as described above. Raf-B activity was also assayed from the same cell lysates to test whether its regulation differed from that of MEKK. Raf-B was immunoprecipitated from the same cell lysates as described above and assayed for its ability to phosphorylate MEK-1 as described above. Forskolin pretreatment abolished the activation of both MEKK and Raf-B by EGF, NGF, and TPA, as measured by their ability to phosphorylate kinase-inactive MEK-1 (Figure 10). Forskolin treatment alone had no appreciable effect on either kinase. These results demonstrate that in addition to Raf-1 and Raf-B, PKA activation inhibits growth factor stimulation of 98 kD PC12 cell MEKK, suggesting the existence of a common regulatory control point for PKA action which lies between or downstream of Ras and upstream or at the level of each of these three kinases.

Example 14. This Example describes the determination of whether a similar or distinct MEK activity is involved in activation of MAPK through G_i protein coupled receptors by measuring MEK activity in cell lysates from thrombin stimulated Rat 1a cells.

Thrombin stimulated cells exhibited a MEK activity which co-fractionated with the major MEK peak detected in EGF stimulated cells. The magnitude of MEK activity from thrombin challenged cells was generally two to three-fold less than that observed with EGF stimulation, which correlates with the smaller MAPK response the present inventors have observed in thrombin challenged cells.

Differential regulation of MEK in Rat 1a and NIH3T3 cells expressing *gip2*, *v-src*, *v-ras*, or *v-raf* led the present inventor to investigate the protein kinases that are putative regulators of MEK-1. Recently, it was shown that Raf-1 can phosphorylate and activate MEK. Raf activation was assayed in the following manner. Cells were serum starved and challenged in the presence or absence of the appropriate growth factors, as described above. Serum starved Rat 1a cells were challenged with buffer alone or with EGF and Raf was immunoprecipitated using an antibody recognizing the C terminus of Raf. Cells were lysed by scraping in ice cold RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Triton X 100, 10 mM sodium pyrophosphate, 25 mM sodium glycerophosphate, 2mM sodium vanadate, 2.1 μ g/ml aprotinin) and were microfuged for 10 min to remove nuclei. The supernatants were normalized for protein content and precleared with protein A Sepharose prior to immunoprecipitation with rabbit antiserum to the C terminus of Raf-1 and protein A Sepharose for 2-3 h at 4°C. The beads were washed twice with ice cold RIPA and twice with PAN (10 mM Pipes, pH 7.0, 100mM NaCl, 21 μ g/ml aprotinin). A portion of the immunoprecipitate was diluted with SDS sample buffer and used

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The immunoprecipitated Raf, in the presence of γ -³²P-ATP, was able to phosphorylate MEK-1. The recombinant MEK-1 used in this assay was kinase inactive to ensure it did not autophosphorylate as is observed with wild type MEK-1. Little or no phosphorylation of MEK-1 by Raf was observed in immunoprecipitates from control cells. EGF challenge clearly stimulated Raf catalyzed phosphorylation of MEK-1; in contrast, thrombin challenge of Rat 1a cells did not measurably activate Raf even though endogenous MEK was clearly activated. EGF stimulated Raf phosphorylation of recombinant MEK-1 by approximately 2.6-fold over basal. Little phosphorylation of MEK by Raf was observed in Raf immunoprecipitates from Gip2 or v-Src expressing Rat 1a cells. EGF stimulation was still capable of activating Raf catalyzed phosphorylation of MEK-1 in these cell lines by 1.8 and 1.4-fold, respectively. The blunting of the EGF response in Gip2 and v-Src expressing cells is likely a result of desensitization of the EGF receptor upon constitutive activation of MAPK. The amount of Raf in the immunoprecipitates was shown to be similar by subsequent SDS-PAGE and immunoblotting using Raf antibody. Since thrombin stimulation of MEK is two to three-fold over basal, at least a 1.5-fold stimulation of MEK phosphorylation is expected if Raf significantly contributed to MEK activation by this growth factor. This level of activation was detectable in the EGF stimulated Gip2 and v-Src expressing cells lines. Thus, it is unlikely that the failure to detect thrombin activation of Raf is due to the sensitivity of the assay. Thrombin stimulation of MAPK is maximal at 3 minutes. Stimulation of Rat 1a cells for 1 or 5 minutes with thrombin did not increase Raf activity.

In NIH3T3 cells, as in Rat 1a cells, EGF activates Raf approximately 2.7-fold, while thrombin does not. V-Raf expressing NIH3T3 cells showed no increase in MEK-1 phosphorylation. This result was unexpected since MEK was clearly activated in v-Raf expressing NIH3T3 cells. Both the p90 and p75 gag-raf fusion proteins in addition to c-Raf-1 were immunoprecipitated from v-Raf NIH3T3 cells by the antisera. P75gag-raf has been shown to exhibit protein kinase activity, but it is possible that the NH₂ terminal gag fusion protein sterically hinders Raf phosphorylation of recombinant MEK-1 in the *in vitro* assay system. Further studies will have to be done to measure v-Raf kinase activity. The results argue that activation of MEK cannot be accounted for exclusively by the activation of Raf.

A Example 15. This Example demonstrates the ability of a PPPSS-trunc¹ and NcoI-trunc² of ^{PPPSS corresponds to} ~~amino acids 211 to 215 of SEQ ID NO:2~~ MEKK protein to activate MAPK activity compared with full-length MEKK protein and a negative control protein.

Example 16: This example describes the preferential activation of JNK by MEKK compared with Raf.

Example 17. This example describes that the phosphorylation of c-Myc transactivation domain in response to MEKK expression activates MYC-GAL 4 transcriptional activity.

Two separate expression plasmids were constructed as follows. The expression plasmid pLNCX was ligated to a cDNA clone comprising *c-myc* (1-103) ligated to *GAL4* (1-147) (Seth et al., pp. 23521-23524, 1993, *J. Biol. Chem.*, Vol. 266) to form the recombinant molecule pMYC-GAL 4. The expression plasmid UAS_G-TK Luciferase (Sadowski et al., pp. 563-564, 1988, *Nature*, Vol. 335) was transfected with either pMYC-GAL 4 or pLU-GAL into Swiss 3T3 cells using standard methods in the art to form recombinant cells herein referred to as LU/GAL cells. Recombinant control cells were also produced by transfecting in pGAL4-Control plasmids containing *GAL4* (1-147) alone in the absence of *c-myc* (1-103).

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cells were incubated with the activity of the 13 indicate the transactivation of the transcription of the gene is not stimulated. A schematic diagram is shown in Figure 1.

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COS cells were transfected with the expression plasmid pCVM5 ligated to cDNA clones encoding either MEKK 1 or MEKK 3 protein, or a control pCVM5 plasmid lacking MEKK cDNA inserts. Forty-eight hours after transfection, the COS cells were lysed and the lysate fractionated on a Mono Q FPLC column using conditions described in Example 4. The fractions were analyzed for tyrosine phosphorylation of MAP kinase-like enzymes using the kinase assay described in Example 4. Expression of MEKK 3 induces tyrosine phosphorylation of p38 MAPK and the p42 and p44 forms of MAPK. MEKK 1, however, only induces weak phosphorylation of p38 MAPK but does induce phosphorylation of p42 and p44 MAPK.

This example describes MEKK-induced apoptosis.

Cells were prepared for the apoptosis studies as follows. Swiss 3T3 cells and REF52 cells were transfected with an expression plasmid encoding β -Galactosidase (β -Gal) detection of injected cells. One set of β -Gal transfected cells were then microinjected with an expression vector encoding MEKK370-738 protein. Another set of β -Gal transfected cells were then microinjected with an expression vector encoding truncated BXB-Raf protein.

A first group of transfected Swiss 3T3 cells and REF52 cells were treated with 50 μ M beauvericin for 6 hours at 37°C. Beauvericin is a compound known to induce apoptosis in mammalian cells. A second group of cells were treated with a control buffer lacking beauvericin. The treated cells were then fixed in paraformaldehyde and permeabilized with

B. MEKK-induced apoptosis

Example 20. This example describes MEKK-induced apoptosis, which is independent of JNK/SAPK activation.

Microinjection

Swiss 3T3 and REF52 cells were plated on acid-washed glass cover slips in Dulbecco's Modified Eagle's Medium (DMEM) and 10% bovine calf serum (BCS) or newborn calf serum (NCS). Cells were placed in DMEM/0.1% calf serum for overnight incubation prior to microinjection and used for injection at 50-70% confluence. Injections were performed with an Eppendorf automated microinjection system with needles pulled from glass capillaries on a vertical pipette puller (Kopf, Tujunga, CA). Cells were injected with pCMV β -gal in the presence or absence of pCMV5MEKK_{COOH} or pCMV5BxBRaf at 20-100 ng/ μ l for each expression plasmid in 100 mM KCl, 5 mM NaPO₄, pH 7.3. Following injection cells were placed in 1% NCS for 12-18 hr (Swiss 3T3) or 42 hr (REF52) prior to fixation with paraformaldehyde and staining. Similar results were obtained when cells were

JNK/SAPK: Activity was measured using GST (glutathione S-transferase)- c-Jun (1-79) BOUND to glutathione-Sepharose-4B (Hibi et al. *supra*). Cells expressing MEKK_{COOH} or control cells were lysed in 0.5% Nonidet P40 (NP40), 20 mM Tris-HCl, pH 7.6, 0.25 NaCl, 3mM EDTA, 3mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2mM sodium vanadate, 20 µg/ml aprotinin and 5 µg/ml leupeptin. Lysates were centrifuged at 15,000 xg for 10 min to remove nuclei and supernatants (25 µg protein) mixed with 10 µl of GST-c-JUN₍₁₋₇₉₎-Sepharose (3-5 µg of GST-c-Jun₍₁₋₇₉₎). The mixture was rotated at 4°C for 1 hr, washed 2x in lysis buffer and 1x in kinase buffer (20 mM Hepes, pH 7.5, 10 mM MgCl₂, 20 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate, 1 mM dithiothreitol, 50 µM sodium vanadate). Beads were suspended in 40 µl of kinase buffer with 10 µCi Of [γ^{32} P] ATP and incubated at 30°C for 20 min. Samples were boiled in Laemmli buffer and phosphorylated proteins resolved on SDS/10% polyacrylamide gels. To verify the selectivity of the JNK/SAPK assay cell lysates were fractionated by Mono Q ion exchange chromatography and each fraction assayed as described above. Fractions were also

immunoblotted with a rabbit antisera recognizing JNK/SAPK. Only fractions containing immunoreactive JNK/SAPK phosphorylated the GST-c-Jun₍₁₋₇₉₎ protein.

p42/44 ERK MAPK: ERK activity was assayed after fractionation of cell lysates on DEAE-Sephacel (Heasley, L.E. et al. (1994) *Am J. Physiol.* 267:F366-F373). Alternatively, ERK activity was assayed following Mono Q ion exchange chromatography as previously described and characterized (Heasley, et al. (1992) *Mol. Biol. Cell.* 3:545-553). The EGF receptor 662-681 peptide was used as a selective substrate for measuring ERK activity (Russell, M. et al. (1995) *Biochemistry.* 34:6611-6615).

p38/Hog-1: Cells were lysed in 1% Triton X-100, 0.5% NP40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20mM NaF, 0.2 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation at 15,000 xg for 5 min. Supernatants (200 µg protein) were used for immunoprecipitation of p38/Hog-1 using rabbit antiserum raised against the COOH-terminal peptide sequence of p38/Hog-1 (CFVPPPLDQEEMES) (Han, J. et al. (1992) *Mol. Endocrinol.* 6:2079-2089) and protein A Sepharose. Immunoprecipitates were washed 1x in lysis buffer, 1x in assay buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM NaCl₂, 2 mM dithiothreitol, 0.1 mM sodium vanadate), resuspended in kinase assay buffer with 20-50 ng of a recombinant NH₂-terminal fragment of ATF-2 as substrate and 20 µCi [γ32P] ATP (Abdel-Hafig, et al. (1992) *Mol. Endocrinol.* 6:2079-2089). For verification of the immunoprecipitation assay lysates were fractionated by Mono Q ion exchange chromatography and each fraction assayed for ATF-2 kinase activity and immunoblotted with anti-p38 antibody. The results demonstrated that p38/Hog-1 containing fractions selectively phosphorylated the recombinant ATF-2 protein.

Competitive Inhibitory Mutant JNK/SAPK and JNKK/SEK-1: The competitive inhibitory JNK/SAPK mutant referred to JNK/SAPK(APF) had the amino acids threonine 183 and tyrosine 185 mutated to alanine and phenylalanine, respectively (Lin et al. (1995) *Science* 268:286-290). These are the sites phosphorylated by JNKK/SEK-1 and required for activation of the JNK/SAPK kinase activity (Lin et al. *supra*; Sanchez, I. (1994) *Nature* 372:794-800). Competitive inhibitory JNKK/SEK-1 was made by mutation of the active site lysine at residue 116 mutated to an arginine (K116R) rendering the protein kinase inactive (Lin et al. *supra*).

A. Expression of activated MEKK induces cell death

For further analysis and comparison cells were microinjected with BxBRaf, a truncated activated form of Raf-1 (Rapp, U.R. (1991) *Oncogene* 6:495-500) that selectively activates the ERK pathway (Kyriakis, J.M. et al. (1992) *Nature* 358:417-421). In microinjected cells, expression of β -gal, MEKK_{COOH} or BxBRaf was demonstrated by indirect immunofluorescence using specific antibodies recognizing each protein. Swiss 3T3 cells and REF 52 cells microinjected with the indicated expression plasmid were fixed and stained only eight hours postinjection to demonstrate that each protein was being expressed in the cytoplasm of the cells. It was apparent with the REF 52 cells expressing MEKK began to undergo a morphological changes relative to β -gal expressing cells.

Table 1: Quantitation of MEKK_{COOH}-induced cell death.

<u>DNA Injected</u>	<u>Cells Injected</u>	<u>Condensed Cells</u>	
β -gal	336	4	(1%)
β -gal+ BxBRaf	175	5	(3%)
β -gal+ MEKK _{COOH}	200	167	(84%)
β -gal+ Kin~MEKK _{COOH}	50	0	(0%)

Swiss 3T3 cells were injected with solutions containing 100 ng/ μ l CMV- β gal in the presence or absence of 100 ng/ μ l of pCMV5-BxEBRaf, pCMV5-MEKKCooH or pCMV5-Kin~MEKK_{COOH} (kinase inactive mutant; 13). Seventeen hours after injection cells were fixed and stained for β -galactosidase activity with X-Gal. Injected cells attached to the coverslip were scored as positive for cell death when they were highly condensed, small round cells.

The results of this experiment demonstrated that expression of MEKK_{COOH} resulted in significant cell death characterized by the dramatic morphological condensation. In contrast, BxBRaf expression did not affect cell viability relative to control cells expressing only β -gal. Approximately 84% of all MEKK_{COOH} injected cells had a highly condensed cellular morphology seventeen hours after injection. This count actually underestimates the number of condensed cells because Swiss 3T3 cells in advanced stages of the cell death response were often nonadherent to coverslips. Some of the nonadherent highly condensed cells could be found to be released from the coverslip into the culture medium, but were not scored in the quantitation. In contrast, fewer than 3% of BxBRaf and 1% of control β -gal injected cells had an altered morphology even after 48-72 hours post-injection.

More dramatic morphological changes in Swiss 3T3 cells also resulted from expression of MEKK_{COOH}. Cytoplasmic shrinkage is evident from the β -gal staining and nuclear condensation is evident in MEKK1 expressing cells stained with propidium iodide. In contrast, cells expressing BxBRaf do not demonstrate any detectable morphological difference from control cells expressing only β -gal. Similar dramatic cytoplasmic shrinkage and nuclear condensation was observed with MEKK_{COOH} expression in REF52 cells, where BxBRaf again had no effect on cytoplasmic and nuclear integrity. To assess if DNA fragmentation was induced by MEKK_{COOH} expression, terminal deoxytransferase (TDT) was used to covalently transfer biotin-dUTP to the ends of DNA breaks *in situ*. Streptavidin-FITC was then used for detection of dUTP incorporated into cellular DNA. Even though Swiss 3T3 cells do not undergo significant DNA degradation and laddering at the nucleosomal level they do generate larger DNA fragments when stimulated to undergo apoptosis (Obeid, L.M. et al. (1993). *Science* 259:1769-1771). The condensed nuclei of MEKK_{COOH} injected cells were highly fluorescent indicating significant DNA fragmentation. It is also apparent that the cytoplasm has become highly condensed and the condensed chromatin is distinct from the cytoplasm. Microinjected cells not yet undergoing cytoplasmic and nuclear condensation in response to MEKK_{COOH} did not incorporate dUTP into their DNA. Thus, expression of MEKK_{COOH} induced all the hallmarks of apoptosis including cytoplasmic shrinkage, nuclear condensation and DNA fragmentation.

Expression of BxBRaf did not induce a response measured by any of the criteria mentioned above. BxBRaf expressing cells displayed a normal flattened morphology similar to β -gal expressing cells or to uninjected cells. Transient BxBRaf expression in Swiss 3T3 cells stimulated ERK activity (not shown) and the transactivation function of the Gal4/ Elk-1 chimeric transcription factor, shown in Figure 15, whose activation is dependent on phosphorylation by Erk members of the MAPK family (Marais, R., Cell 73:381-393; Gille, et

al. (1995) EMBO J. 14:951-962; Price, M.A., et al. (1995) EMBO J. 14:2589-2601). Cumulatively, the results indicate that activation of the Raf/ERK pathway does not induce the cytoplasmic and nuclear changes observed with MEKK.

B. Induction of activated MEKK sensitizes Swiss 3T3 cells to UV-induced apoptosis

Because stable expression of MEKK_{COOH} appeared to inhibit clonal expansion of Swiss 3T3 cells under G418 drug selection, clones were isolated having inducible expression of the kinase. The Lac Switch expression system (Stratagene) was used to control the expression of MEKK_{COOH}. Several independent clones were isolated and their properties analyzed in the presence or absence of IPTG-induced expression of MEKK_{COOH}. The parental LacR⁺ clone expressing only the Lac repressor was used as the control. Clones expressing inducible MEKK_{COOH}, as determined using an antibody recognizing the extreme COOH-terminus of MEKK, showed a small increase in the number of cells having a condensed cytoplasmic and nuclear morphology relative to control cells even in the absence of IPTG-induced MEKK_{COOH}. This is probably due to a basal level of MEKK_{COOH} expression in uninduced cells. The addition of IPTG to the culture media induced the expression of MEKK_{COOH} and resulted in an increase in cells having the condensed morphology relative to the control IPTG-treated LacR⁺ clone. However, MEKK_{COOH} expressing cells did not growth arrest and only a fraction of the cells assumed a condensed morphology as dramatic as what was observed with microinjection of the MEKK_{COOH} expression plasmid. This maybe related to selection of cells during the cloning procedure that adapted to a low, constitutive level of MEKK_{COOH} expression. Interestingly, no clones were isolated from a total of one hundred fifty that were analyzed that had a significant constitutive MEKK_{COOH} expression measured by immunoblotting. In addition, the level of MEKK_{COOH} expression following IPTG induction is certainly less than that achieved with nuclear microinjection.

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It was found that IPTG-induced MEKK_{COOH} expression stimulated signal transduction pathways that made the cells significantly more sensitive to stresses that induce cell death. For example, cells expressing MEKK_{COOH} were highly sensitive to ultraviolet irradiation. Two hours after exposure to ultraviolet irradiation greater than 30% of the MEKK_{COOH} expressing cells became morphologically highly condensed and appeared apoptotic. In contrast, the population of uninduced cells showed no increase in condensed apoptotic-like cells at this time point (Figure 16). Thus, overnight induction of MEKK_{COOH} expression modestly increased the basal index of morphologically condensed cells and primed the cells for apoptosis in response to UV irradiation. The results indicate that MEKK-regulated signal transduction pathways enhance apoptotic responses to external stimuli.

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C. Expression of MEKK_{COOH} stimulates JNK/SAPK and the transactivation of c-Myc and Elk-1 The ability of MEKK_{COOH} but not BxBRaf expression to induce cell death indicates that each kinase regulates different sequential protein kinase pathways. Cells were incubated for 17 hours in the absence or presence of IPTG and assayed for JNK/SAPK activity. The induction of MEKK_{COOH} expression in Swiss 3T3 cells, as predicted, stimulated JNK/SAPK activity but did not activate either ERK or p38/Hog1 activity as shown in Figures 17 and 18. The results indicate that induction of MEKK_{COOH} results in the activation of JNK/SAPK which phosphorylates GST-c-Jun. Because known substrates for JNK/SAPK are transcription factors, we assayed MEKK_{COOH} inducible clones for transactivation of specific gene transcription. Chimeric transcription factors having the Gal4 DNA binding domain and the transactivation domain of c-Myc, Elk-1 or c-Jun were used for assay of MEKK_{COOH} signaling using a Gal4 promoter-luciferase reporter gene (Hibi et al. *supra*; Sadowski, I et al. (1988) *Nature* 335:563-564; Gupta et al. *supra*; Marais et al. *supra*). Surprisingly, IPTG-induced stable expression of MEKK_{COOH} markedly activated the transactivation function of c-Myc and Elk-1 but had little effect on Gal4/Jun activity as illustrated in Figure 18. This result was unexpected since MEKK_{COOH} transient expression stimulated Gal4/Jun activity, indicating that transient expression of MEKK_{COOH} was capable of transactivating c-Jun function in Swiss 3T3 cells. In addition, the JNK/SAPK activity stimulated by IPTG-induction of MEKK_{COOH} correlated with the characterized JNK/SAPK enzyme by fractionation on Mono Q FPLC. Thus, MEKK_{COOH} expression in stable clones achieved with IPTG-induction selectively regulated Gal4/Myc and Gal4/Elk-1 but not Gal4/Jun even though JNK/SAPK was activated.

The failure of IPTG-induced MEKK_{COOH} expression to activate Gal4/Jun may be related to the multiple c-Jun NH2-terminal phosphorylation sites involved in regulating c-Jun transactivation. Serines 63 and 73 and threonines 91 and 93 are apparent regulatory phosphorylation sites in c-Jun (Kyriakis et al. (1994) *Nature* 369:156-160; Derijard, B et al. (1994) *Cell* 76:1025-1037; Pulverer et al. (1991) *Nature* 353:670-674; Papavassiliou, et al. (1995) *EMBO J.* 14:2014-2019). Both clusters are proposed to be sites of phosphorylation for ERKs and JNK/SAPKs (Papavassiliou et al. *supra*). Transient transfection of MEKK_{COOH} activates JNK/SAPK but also activates ERKs (Lange-Carter et al. *supra*). In contrast IPTG-induction of MEKK_{COOH} results in the activation of JNK/SAPK but not Erks. The difference in regulation of c-Jun transactivation may be related to the differential phosphorylation of these sites by JNK/SAPK and ERKs.

Expression of activated Raf in Swiss 3T3 cells stimulated Elk-1 transactivation, but not c-Myc or c-Jun transactivation. This result indicates that Elk-1 transactivation alone does not mediate the cell death response in fibroblasts observed with MEKK_{COOH}. Cumulatively, the findings demonstrate that induction of MEKK_{COOH} expression enhances cell death

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The cell death response to MEKK_{COOH} also appeared to be largely independent of JNK/SAPK. In several experiments, expression of JNK/SAPK(APF) alone had no demonstrative effect on Swiss 3T3 cells. The expressed JNK/SAPK(APF) was localized in both the cytoplasm and nucleus while β -gal expression was restricted to the cytoplasm. Co-expression of JNK/SAPK(APF) with MEKK_{COOH} did not block MEKK_{COOH}-induced cytoplasmic shrinkage and cellular condensation. A 20-fold lower concentration of MEKK_{COOH} still induced the cytoplasmic shrinkage characteristic of apoptosis in microinjected Swiss 3T3 cells. Co-microinjection of a 30-fold greater concentration of JNK/SAPK(APF) plasmid relative to the MEKK_{COOH} plasmid did not affect the MEKK_{COOH}-mediated cell death response. Cells undergoing a dramatic cytoplasmic shrinkage. Because of the low amount of MEKK_{COOH} expression plasmid used, the cell condensation response was slower in onset. The percentage of MEKK_{COOH} microinjected cells committed to cytoplasmic shrinkage and cellular condensation and the timing of this response was the same in the presence or absence of JNK/SAPK(APF). In addition, the competitive inhibitory 'mutant K116RJNKK/SEK-1, the kinase immediately upstream of JNK/SAPK which phosphorylates and activates JNK/SAPK (Lin et al *supra*; Sanchez, I (1994) *Nature* 372:794-800) also unable to attenuate MEKK_{COOH} induced cell death. Expression of JNK/SAPK(APF) or K116RJNKK/SEK-1 alone had no measurable effect on the morphology of Swiss 3T3 cells (not shown). Thus, MEKK_{COOH} induces cell death via the regulation of signal pathways that

Recently, it was shown that dominant negative c-Jun could protect neurons from serum deprivation-induced apoptosis (Ham, J. et al. (1995) *Neuron* 14:927-939). It was proposed that the dominant negative cJun inactivated c-Jun and prevented an attempt by the post mitotic neurons to enter an abortive cell cycle progression that triggered a cell death program. Thus, dominant negative c-Jun was believed to maintain the neurons in stringent growth arrest. At first glance, the protective effect of dominant negative c-Jun seems

Expression of MEKK_{COOH} increased the transactivation of c-Myc and Elk-1 in Swiss 3T3 cells. c-Myc has been shown to be required for apoptosis in lymphocytes (Fanidi, A et al. (1994) Nature 359:554-556; Janicke, R.U. et al (1994) Mol. Cell. Biol. 14, 5661-5670; Shi et al. (1992) Science 257:212-214), to induce apoptosis when overexpressed in growth factor-deprived fibroblasts (Harrington, E. A. et al. (1994) EMBO J. 13:3286-3295); Askew, D.W., et al. (1991) Oncogene 6:1915-1922; Evan, G.I. et al. (1992) Cell 69:119-128), and to enhance TNF-mediated apoptosis (Kleefstrom, J., et al. (1994) EMBO J. 13:5442-5450). The requirement of c-Myc for apoptosis is not understood mechanistically, but c-Myc is proposed to transcriptionally activate an apoptotic pathway (Harrington, E.A. et al. (1994) EMBO J. 13:3286-3295); Askew et al. *supra*; Evan et al. *supra*; Janicke et al. *supra*; Shi et al. *supra*). The activation of Elk-1 by MEKK_{COOH} induction in Swiss 3T3 cells correlates best with the stimulation of JNK/SAPK. Recently, it was found that JNK/SAPK in addition to Erks phosphorylated and activated Elk-1 consistent with our findings (Whitmarsh, A.J. et al. (1995) Science 269:403-407). In contrast, we demonstrate that c-Jun is not significantly activated in MEKK_{COOH} expressing cells. These findings are provocative because they indicate that MEKK-stimulated JNK/SAPK activation preferentially regulated Elk-1 and not c-Jun. A second signal in addition to JNK/SAPK may be required for c-Jun transactivation in cells (Papavassiliou, A.G., et al. (1995) EMBO J. 14:2014-2019). There does not seem to be a proposed role for Elk-1 in inducing an apoptotic response, but serum deprivation-induced apoptosis of Swiss 3T3 cells results in the increased expression of early cell cycle genes consistent with an increased SRF/SRE activity associated with elevated Elk-1 activity (Pandey, S. and Wang, E. (1995) J. Cell. Biochem. 58:135-150). The induction of apoptosis in several cell types does not appear to require transcription, but the use of inducible cell lines and plasmid microinjection experiments do not facilitate testing whether MEKK_{COOH} can induce cell death in the absence of transcription. In cells where transcription is not necessary for the induction of apoptosis it is likely that proteins required for apoptosis are already expressed and may be post translationally regulated by sequential protein kinase pathways

In Jurkat cells, a human T cell line, Fas-induced apoptosis has been proposed to involve a ceramide stimulated, Ras-dependent signaling pathway (Gulbins, E., et al. (1995) *Immunity* 2:34351). We recently demonstrated that MEKK activity can be stimulated by Ras and that MEKK1 physically binds to Ras in a GTP-dependent manner (Russell, M. et al. (1995) *J. Biol. Chem.* 270:11757-11760; Winston, B.W., et al. (1995) *Proc. Natl. Acad. Sci. USA* (1995) 92:1614-1618). The ability of MEKK to regulate an apoptotic-like cell death response suggests it is a candidate component for the ceramide regulated apoptotic pathway.

The importance of our observations describing the involvement of MEKK regulated sequential protein kinase pathways in physiologically relevant signaling leading to cell death is supported by several findings. First, MEKK_{COOH} induces or enhances a cell death response in the presence of 10% calf serum, indicating that growth factor deprivation is not a prerequisite for MEKK-induced cell death. This is similar to TNF α , Fas and ceramide-mediated apoptosis which proceeds in high serum. Thus, the involvement of MEKK in cell death responses is not simply to activate a subset of growth factor stimulated signaling events causing an aborted cell cycle-induced apoptosis that would normally be prevented by serum factors. Second, the enhanced cell death to ultraviolet irradiation indicates that expression of MEKK_{COOH} may activate signals that potentiate stresses to the cell. This finding indicates that MEKK-regulated signal transduction pathways integrate with cellular responses involved in mediating apoptosis, that ultraviolet irradiation likely activates additional pathways and that MEKK_{COOH}-mediated signaling synergizes with the ultraviolet response to accelerate apoptosis. Third, MEKK stimulated sequential protein kinase pathways independent of ERK, JNK/SAPK, p38/Hog1 and c-Jun transactivation that can stimulate c-Myc transactivation. These results indicate that MEKK-regulated pathways traverse the cytoplasm to regulate as yet undefined protein kinases that activate cMyc in the nucleus. The regulation of c-Myc activity is a unique function of MEKK signaling and one that we postulate is likely to contribute to the cell death response. Serum deprivation significantly induces JNK/SAPK activation in several cell types including Swiss 3T3 cells. Similarly, TNF α stimulates a JNK/SAPK pathway (Minden et al. (1994) Science 266:1719-1723) and we have recently demonstrated TNF α stimulation of MEKK activity in mouse macrophages (Winston et al. supra). c-Myc overexpression has been shown to enhance TNF α receptor stimulation of apoptosis (White et al. (1992) Mol. Cell. Biol. 12:2570-2580). These findings are consistent with a linkage between TNF α receptor signaling, MEKK and c-Myc.

Example 21. This example illustrates that TNF and expression of MEKK1_{COOH} synergize to induce apoptosis in cells.

Example 22. This Example describes regulation of MAPK activity by both MEKK and Raf protein.

Referring to Figure 21, both MEKK and Raf overexpression in COS 1 cells resulted in similar levels of stimulation of MAPK activity over basal levels.

Example 23. This example demonstrates the ability of COS cell-expressed MEKK1 proteins to bind to GST-Ras^{V12}.

COS cells were transiently transfected by the DEAE-dextran protocol as generally described in Example 3. Cos cells were transfected with: (1) p-MEKK1 containing a nucleic acid molecule encoding MEKK1 as described in Lange-Carter et al. (*Science* 260:315-319, 1994); (2) p-MEKK_{NH2} containing a nucleic acid molecule that encodes a 858 base pair PvuII(682)-NcoI(1541) restriction digest fragment of the amino terminus of MEKK1 ligated into pCMV5; (3) p-MEKK_{COOH} containing a nucleic acid molecule that encodes a 1435 base pair NcoI(1541)-SspI(2976) restriction digest fragment that includes the entire kinase domain of MEKK1 ligated into pCMV5; (4) pCMV5 without insert; or (5) p-C4Raf containing a

COS cells expressing the various MEKK1 proteins were lysed in EB (1% Triton X-100, 10mM Tris HCl [pH 7.4], 5mM EDTA, 50mM NaCl, 50mM NaF, 0.1% bovine serum albumin, 0.2 U/ml aprotinin, 1mM phenylmethylsulfonyl fluoride and 2mM Na₃ VO₄). The lysates were separated into two equal parts for separate binding reactions. Half of the lysate was incubated with GST agarose (1.5μg) while half of the lysate was incubated with GST-Ras^{V12} agarose (1.5μg) (purchased from UBI) for 1 hr at 4°C. The GST-Ras^{V12} was preincubated at 30°C for 30 min with 1mM nucleotide (GDP or GTPγS). The nucleotide binding reaction was stopped by adding MgCl₂ to a final concentration of 20mM. After the 1 hr binding reaction the agarose beads were pelleted at 2000 rpm for 2 min and washed 3 times with PBS + 1.0% Triton X-100. The washed agarose beads were boiled in Laemmli SDS sample buffer and the proteins resolved by SDS polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose for immunoblotting with antibodies specific for an NH₂ terminal fusion protein (described in Example 1) or a COOH terminal peptide (described in Example 1). C4Raf binding was detected using an antibody specific for Raf described in Example 8.

Initial immunoblotting results using anti-Raf antibodies demonstrated that C4Raf bound to GST-Ras^{V12}(GTPγS) agarose but not to the GST agarose control. Additionally, no Raf immunoreactive proteins were detected bound to Ras from COS cells transfected with pCMV5. These results indicated that the Ras binding assay was functional.

Immunoblotting results using anti-MEKK antibodies indicate that protein encoded by p-MEKK1 (MEKK1) transiently expressed in COS cells was capable of binding GST-Ras^{V12} in a GTP dependent manner. MEKK1 from COS cell lysates bound to GST-Ras^{V12}(GTPγS), while little binding to GST-Ras^{V12}(GDP) was detectable. With the conditions used, MEKK1 binding to GST-Ras^{V12}(GTPγS) was at least 5-fold greater than the binding to GST-Ras^{V12}(GDP). No detectable MEKK1 was bound to GST.

The domain critical for the binding of MEKK1 to Ras was then identified. The protein encoded by p-MEKK_{COOH} (MEKK_{COOH}) bound to GST-Ras^{V12} in a GTP dependent manner. Little MEKK_{COOH} bound to GST-Ras^{V12}(GDP). No detectable MEKK_{COOH} was bound to GST. In addition, when protein encoded by p-MEKK_{NH2} (MEKK_{NH2}) was expressed in COS cells, no binding to Ras was detected. In contrast to the ability of Raf-1 to bind to Ras through its amino terminus, MEKK_{NH2} failed to bind GST-Ras^{V12}(GTP γ S) even though the protein was expressed to similar levels as MEKK1 in the same experiment. Thus, GST-Ras^{V12} binds to MEKK1 at a site located within the COOH-terminal catalytic domain of MEKK1.

A construct encoding the kinase domain of a Rat MEKK1 cDNA (95% identical to mouse MEKK1) with a N-terminal hexahistidine tag (referred to herein as MEKK_{COOH}-His; provided by Dr. Melanie Cobb, Department of Pharmacology, University of Texas Southwestern Medical School, Dallas, TX) was expressed in bacteria and soluble active enzyme was purified on Ni₂+NTA agarose according to the method generally described in Gardner et al. (*Methods of Enzymology* 238:258-270, 1994) Purified recombinant MEKK_{COOH}-His was incubated with either GST or GST-Ras^{V12} in PAN buffer (10mM PIPES [pH 7.0], 100mM NaCl, 0.2 U/ml aprotinin) for 1 hr at 4°C. The agarose beads were pelleted and washed 3 times in PAN buffer. The washed agarose beads were then incubated in kinase buffer (20mM PIPES [pH 7.0], 10mM MnCl₂, 40μCi[γ³²P]ATP, 20μg/ml aprotinin) containing 100ng recombinant kinase inactive MEK1 as substrate in a final volume of 150 μl, at 30°C for 20 min. To test the direct interaction of MEKK1 with the effector domain of Ras, samples were prepared by pre-incubating the agarose beads with either 100 μM of Ras peptide consisting of residues 17-42 of H-Ras or 100 μM of Ras control peptide ([D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹] substance P peptide for 1 hr at 4°C prior to addition of the MEK1 substrate. A control reaction containing wild-type MEKK1 which autophosphorylates, served as a marker for the MEKK1 substrate. Reactions were terminated by addition of 5X Laemmli SDS sample buffer, boiled and resolved by SDS-PAGE.

The results indicate that there was direct binding of Ras-GTP γ S to purified MEKK_{COOH}-His as measured by the increased phosphorylation of KM MEK1 using GST-Ras^{V12}(GTP γ S) beads incubated with recombinant MEKK_{COOH}-His. The interaction between Ras and MEKK_{COOH}-His was GTP dependent because essentially no KM MEK1 phosphorylation could be detected with GST-Ras^{V12}(GDP) beads incubated with recombinant MEKK_{COOH}.

The results indicate that the presence of Ras effector peptide prevented the binding of GST-Ras^{V12}(GTPγS) agarose to MEKK_{COOH}-His, thereby preventing the phosphorylation of KM MEK1 substrate present in the sample. MEKK_{COOH}-His was able to bind to GST-Ras^{V12}(GTPγS) in the presence of buffer alone or in the presence of a control peptide ([D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹] substance P peptide), resulting in the phosphorylation of KM MEK1 substrate.

Taken together, the results described in Examples 22 and 23 demonstrate that MEKK1 is a Ras effector and selectively binds to Ras in a GTP dependent manner. In addition, the binding of MEKK1 to Ras *in vitro* is direct and occurs via the COOH terminal region of MEKK1 that encodes the catalytic kinase domain.

Example 25. This example demonstrates the cloning of MEKK4.1 and MEKK4.2, a splicing variant of MEKK4.

The degenerate primers GA(A or G)(C or T) TIATGGCIGT^{AA}AMINO ACIDS(A or G)CA_A(sense) and TTIGCICC(TorC)TTIAT(A or G)TCIC(G or T)(A or G)TG_A(antisense) were used in a polymerase chain reaction (PCR) using first strand cDNA generated from polyadenylated RNA prepared from NIH 3T3 cells. The PCR reaction involved 30 cycles (1 minute, 94°C/2 minutes, 52°C/3 minutes, 72°C) followed by a 10 minute cycle at 72°C. A band of approximately 300 bp was recovered from the PCR mixture and the products cloned into pGEM-T (Promega). The PCR cDNA products were sequenced and compared to the MEKK1 sequence. A unique cDNA sequence having significant homology to MEKK1 cDNA was identified and used to screen an oligo dT primed mouse brain cDNA library (Stratagene). The λ phage library was plated and DNA from plaques transferred to hybrid-N filters (Amersham) followed by UV-crosslinking of DNA to the filters. Filters were pre-hybridized for 2 hours and then hybridized overnight in 0.5 M Na₂H₂PO₄ (pH 7.2), 10% bovine serum albumin, 1 mM EDTA, 7% SDS at 68°C. Filters were washed 2x at 42°C with 2X SSC, 1x with 1X SSC and 1x with 0.5X SSC containing 0.1% SDS (1X SSC is 0.15 M NaCl, 0.015M sodium citrate, pH 7.0). Positive hybridizing clones were purified and sequenced. To resolve GC-rich regions cDNAs were subcloned into M13 vectors (New England Biolabs) and single strand DNA sequenced. In all cases both strands of DNA were sequenced. Clones were truncated at the 5'-region and were therefore not full length in the coding region. To obtain the 5' region of MEKK4 poly RNA was isolated and primers from the partial cDNA used for reverse transcription. cDNAs were generated using the RACE procedure and sequenced. The 5' region of MEKK4 with upstream in frame stop codons was obtained and ligated to the partial MEKK4 cDNA to give a full length MEKK4 cDNA having an open reading frame of 1597 codons.

Example 26. This example demonstrates the differential expression of MEKK4.2.

RNA was isolated from the indicated tissues of a Balb/c mouse tissues. RNA was isolated from the indicated tissues of a Balb/c mouse, resolved on an agarose gel, transferred to nitrocellulose paper and hybridized with ³²P-labeled MEKK4.2 cDNA probe. A single mRNA band approximately 5.8 kb is hybridized with the labeled MEKK4.2 probe.

Example 27. This example demonstrates that the MEKK4 kinase domain activates c-Jun kinases activity.

COS cells were transfected with pCMV5 expression plasmid encoding no cDNA insert (control), full length MEKK4 or the truncated MEKK4 encoding only the catalytic kinase domain. The truncated MEKK4 kinase domain is constitutively active when

expressed in COS cells. The MEKK1 kinase catalytic domain, and MEKK2 and -3 also activate the c-Jun kinase pathway (see Figure 22).

Example 28. This example demonstrates that MEKK4 does not activate p42/p44 MAP kinases (ERK1 and ERK2) activity.

COS cells were transfected with pCMV5 expression plasmid encoding no cDNA insert (control), full length MEKK4, the truncated MEKK4 encoding only the catalytic kinase domain or the MEKK1 catalytic domain. The MEKK1 catalytic domain but not the MEKK4 catalytic domain is capable of activating ERK1 and ERK2 (see Figure 23).

Example 29. This example demonstrates that MEKK4 interacts with Cdc42/Rac.

GST fusion proteins encoding Cdc42 or Rac loaded with either GTPys or GDP were incubated with MEKK4 using previously described methods (Russell, M. et al. (1995) J. Biol. Chem. 270:11757-11760). The source of MEKK4 was either from a Cos cell transient transfection or a recombinant MEKK4 protein expressed in E. coli. The recombinant MEKK4 protein was truncated to express residues from 1261-1597 of the full length protein. A GST fusion protein of Ha-Ras was used as a control. The MEKK4 protein was incubated for 1 hr at 40C with either GST-Cdc42, GST-Rac or GST-Ras bound to glutathione-Sepharose beads. Each GSTfusion protein had GTPys or GDP bound to the Cdc42, Rac or Ras moiety of the fusion protein. Following the incubation the beads were washed extensively and the bound proteins removed in SDS-Laemmli buffer and resolved by SDS-PAGE using 10% acrylamide gels. The proteins were transferred to nitrocellulose and immunoblotted using a MEKK4 specific antibody recognizing the extreme COOH-terminus of MEKK4. MEKK4 specifically bound to GST-Cdc42 and GST-Rac in the GTP γ S form. The GDP bound forms of GST-Cdc42 and GST-Rac bound less than 10% of the MEKK4 bound in the presence of GTPys. MEKK4 did not bind significantly to GST-Ras in either the GTP γ S or GDP bound form.

The sequence ⁵⁹⁹⁻⁶²¹ IIGQVCDTPKSYDENVHVGLRKV (residues ¹³⁰⁶⁻¹³²⁷ of the MEKK4 sequence) was synthesized as a GST-fusion protein by standard PCR techniques. The GST-fusion peptide bound Cdc42 and Rac in the GTP γ S bound form. This fusion protein did not bind Ras using the procedures described above.

Example 30.

Tumor necrosis factor α (TNF α) is a multifunctional cytokine secreted primarily by activated monocytes (Tracy, K.J., and Cerami, A. (1993) *Annu. Rev. Cell Biol.* 9:317-343). It has a wide range of biological activities depending upon cell type, stage of differentiation and transformation state. TNF α acts as a growth factor for fibroblasts (Vilcek, J., et al. (1986) *J.*

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Exp. Med. 163:632-643; Victor, I., et al. (1993) *J. Biol. Chem.* 268:18994-18999), is cytotoxic towards certain cells and tumors (Larrick, J. W., and Wright, S.C. (1990) *FASEB J.* 4:3215-3216), induces monocyte differentiation of the human HL-60 myeloid leukemia cell line (Trinchieri, G., et al. (1986) *J. Exp. Med.* 164:1206-1225; Kim, M., et al. (1991) *J. Biol. Chem.* 266:484-489), represses adipocyte (Torti, F.M., et al. (1985) *Science* 229:867-869) and myoblast differentiation (Miller, S.C., et al. (1988) *Mol. Cell. Biol.* 8:2295-2301), and mediates endotoxic shock (Tracey, K.J., et al. (1986) *Science* 234:470-474). The peiotropic effects of this cytokine make it an important mediator in processes as diverse as proliferation, differentiation and cytotoxicity.

TNF α exerts these responses by binding to two cell surface receptore, the 55 kD TNFR (p55 TNFR) and the 75kD TNFR (p75 TNFR) (Loetscher, H., et al. (1990) *Cell* 61:351-359; Schall, T.J., et al. (1990) *Cell* 61:361-370; Smith, C.A., et al. (1990) *Science* 248:1019-1023; Heller, R.A., et al. (1990) *Proc. Natl. Acad. Sci. (USA)* 87:6151-6155). The receptors are single transmembrane spanning glycoproteins present on almost all cells analyzed (Kull, Jr., et al. (1985) *Proc. Natl. Acad. Sci (USA)* 82:5756-5760; Lewis, M., et al. (1991) *Proc. Natl. Acad. Sci. (USA)* 88:2830-2834). The extracellular domain of the p55 TNFR is homologous to the extracellular domains of the low affinity nerve growth factor receptor, the Fas/APO1 receptor, CD40, OX40, and CD27. The p55 TNFR and Fas share a 65 residue homology region in the cytoplasmic domains (Tartaglia, L.A., and Goeddel, D.V. (1992) *Immunol. Today* 13:151-153; Smith, C.A., et al. (1994) *Cell* 76:959-962) which deletion studies have implicated in the TNF α signaling cascade leading to apoptosis (Itoh, N., and Nagata, S. (1993) *J. Biol. Chem.* 268:10932-10937; Tartaglia, L.A., et al. (1993) *Cell* 74:845-853). Most of the known TNF α responses occur by activation of the p55 TNFR. However, thymocyte proliferation is associated with p75 TNFR and eytotoxicity may be a function of p75 TNFR acting alone or in concert with the p55 TNFR (Heller, R.A., et al. (1992) *Cell* 70:47-56).

Apoptosis involves the activation of a specific suicide program within a cell. It occurs when a cell initiates a series of biochemical and morphological events which result in nuclear disintegration and eventual fragmentation of the dying cell into a cluster of membrane-bound apoptotic bodies (Kerr, J., Wyllie, A., and Currie, A. (1972) *Br. J. Cancer* 26:239-257). Apoptosis is responsible for such diverse activities as the elimination of cells during normal embryological development and determination of the immune receptor repertoire (Raff, M.C. (1992) *Nature* 356:297-300; Krammer, P.H., et al. (1994) *Curr. Opin. in Immunol.* 6:279-289; Green, D.R., and Scott, D.W. (1994) *Curr. Opin. in Immunol.* 6:476-487). Apoptosis can be triggered in multiple ways, but it is not yet known whether different

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In many peptide-hormone receptor systems signal transduction to the nucleus involves the sequential activation of protein kinases. The extracellular response kinase (ERK) group of mitogen-activated protein kinases (p42 and p44 MAPK) are activated by growth factors via a Ras/Raf dependent signal transduction pathway (Davis, R.J. (1993) *J. Biol. Chem.* 268:14553-14556; Cano, E. and Mahadevan, L. (1995) *Trends Biochem. Sci.* 20:117-122). In contrast, the JNK/SAPK (Jun kinase/stress-activated protein kinase) members of MAPKs are activated by proinflammatory cytokines and environmental stresses (Devary, et. al. (1992) *Cell* 71:1081-1091; Hibi, M., et al.(1993) *Genes & Development* 7:2135-2148; Sluss, H., et al. (1994) *Mol. Cell. Biol.* 14:8376-8384; Kyriakas, J.M., et al. (1994) *Nature* 369:156-160; Minden, A., et al. (1994) *Mol. Cell. Biol.* 14:6683-6688).

Materials and Methods

Cell lines and culture. L929 cells (ATCC CCL1) were maintained in Dulbecco's modified Eagle's medium with 5% newborn calf serum and 5% bovine calf serum (BCS) supplemented with 100 ug/ml streptomycin and 100U/ml penicillin. The cells were grown in 10cm dishes at 37°C in 7.5% CO₂. Cells were made quiescent where indicated by incubation in Dulbecco's modified Eagle's medium and 0.1% bovine serum albumin for 24 h. Recombinant murine TNF α and recombinant human bFGF (147aa) were from R&D Systems, Minneapolis, MN. Cells were pretreated where indicated with the MEK-1 inhibitor PD#098059 (Parke-Davis Pharmaceutical Corp. Ann Arbor, MI) for 1 h at 37°C. Cells were

Neutral Red Assay Uptake of the dye neutral red was used as one measure of cell viability following cytokine or growth factor treatment (Finter, N.B. (1969) *J. Gen Virol.* 5:419-427). 1.5×10^4 - 2.5×10^5 L929 cells/well were plated in 12 well tissue culture dishes in 1.25 ml of media. Cells were treated for 15-20 hr with various concentrations of TNF α and/or bFGF. 2.5 μ l of 1% neutral red was added to the wells and incubated for 2 hr at 37°C PBS. The neutral red was extracted with 1.0 ml of 50% ethanol, 50 mM Na-citrate pH 4.2 and absorbency was measured at 540 mM.

Cell transfections L929 cells were transfected by CaPO₄ (Ausubel, F. (1994) *Current Protocols in Molecular Biology* Vol. 1, pp. 9.1.1-9.1.4, John Wiley & Sons, Inc., New York) with the vector 3'SS (Stratagene, La Jolla, CA) expressing the LACI repressor. Stable clones were selected in 200 µg/ml hygromycin (Calbiochem, La Jolla, CA) and screened for LACI expression by indirect immunofluorescence using rabbit anti-sera to LACI (Stratagene, La

Immunoblotting 100 µg of cell lysate was fractionated by SDS PAGE (12.5% acrylamide) and blotted to nitrocellulose in 10 mM CAPS, pH 11, 20% MeOH using a Transphor apparatus (Hoeffer, San Diego, CA) for 1 hr at 1 amp. Blots were blocked in 5% powdered milk in Tris-HCl, pH 7.5 buffered saline. Ras was detected with Y-13259 anti-Ras monoclonal antibody (Fruth, M.E., Davis, L.J., Fleurdelys, B., and Skolnick, E.M. (1982) *J. Virol.* 43:294-304) followed by enhanced chemiluminescence (Amersham, Chicago, IL) using HRP-anti-mouse IgG (BioRad, Richmond, CA).

Results

bFGF protects L929 from TNF α -mediated apoptosis TNF α activates a cell death program resulting in the apoptosis of L929 cells (Feshel, K., Kolb-Bachofen, V., and Kolb, H. (1991) *Am. J. Pathol.* 139:251-254). Fig. 24A shows that treatment of L929 cells overnight with TNF α resulted in substantial cell death using the neutral red assay as a measure of viable cells (see Methods). The time course of cell death was dependent on the concentration of TNF α . Treatment with 10 ng/ml TNF α resulted in greater than 40% of the L929 cells being apoptotic in 15 hr; 1 ng/ml TNF α required 24-48 hr to induce a similar level of L929 cell death (not shown). Serum and growth factor withdrawal induces apoptosis in several cell systems (Oppenheim, R.W. (1991) *Annu. Rev. Neurosci.* 14:453-501; Kinoshita, T., et al.(1995) *EMBO J.* 14:266-275), indicating that growth factors have a protective effect against apoptosis. Consistent with this observation was our finding that bFGF affected TNF α mediated apoptosis (Fig. 24B). Incubation of L929 cells with TNF α in the presence of bFGF

was effective at blocking TNF α -mediated cell death. The protective effect of bFGF was not simply due to an increased proliferative response of L929 cells, because bFGF in the absence of TNF α did not measurably increase cell number (Fig. 24B).

Regulation of JNK and MAPK by TNF α and bFGF TNF α has been previously shown to activate p24/p44 MAPK in L929 cells (Van Lint, J., Agostinis, P., Vandevoorde, V., Haegeman, G., Fiers, W., Merlevede, W., and Vandenheede, J. (1992) *J. Biol. Chem.* 267:25916-25921) but recent studies have indicated that TNF α is a potent activator of the Jun kinase (JNK) members of the MAPK family (Sluss, H., et al. (1994) *Mol. Cell. Biol.* 14:8376-8384; Kyriakas, J.M., et al. (1994) *Nature* 369:156-160; Westwick, J., Weitzel, C., Minden, A., Karin, M., and Brenner, D. (1994) *J. Biol. Chem.* 269:26396-6401). Analysis of the time course and dose response of TNF α on L929 cells demonstrated significant differences in the activation of JNK and p42/p44 MAPK activity. Extracts from TNF α -treated versus control L929 cells were assayed for JNK activity using GST-c-Jun(1-79) as substrate. TNF α induced a transient increase in JNK activity that peaked at 10-15 min and returned to two-fold above basal JNK activity 1-2 hr post-stimulation. Maximal JNK activation was achieved at 1 ng/ml TNF α and 0.1 ng/ml TNF α activated JNK greater than four-fold. TNF α stimulation of p42/p44 MAPK activity was slightly more rapid than JNK activation, reaching maximal stimulation in 5-10 min that returned to near basal levels by 30 min (Fig. 25A). The dose-response curve for p42/p44 MAPK activation is dramatically shifted to higher TNF α concentrations than that for JNK (Fig. 25B). Greater than 10 ng/ml TNF α was required to stimulate p42/p44 MAPK 2-3 fold; at 1 ng/ml TNF α the MAPK activity was stimulated only 20% above basal, a concentration of TNF α that gave maximal JNK activation. Thus, TNF α preferentially regulates the JNK pathway relative to p42/p44 MAPK in L929 cells. These findings indicate that the localized concentration of cytokines such as TNF α will determine the selectivity and magnitude of cellular JNK and p42/p44 MAPK responses.

In contrast to proinflammatory cytokines such as TNF α , growth factor receptor tyrosine kinases are generally mitogenic in fibroblasts and stimulate the p42/p44 MAPK pathway. The bFGF receptor possesses intrinsic tyrosine kinase activity and is present on L929 cells. Fig. 26 demonstrates that bFGF stimulates a robust activation of MAPK in L929 cells. Concentrations of 0.25-0.5 ng/ml of bFGF gave maximal stimulation of MAPK activity. Fractionation of stimulated cell lysates by MonoQ fast pressure liquid chromatography indicated that both p42 and p44 MAPK were activated by bFGF (not shown). Activation of the MAPK pathway by tyrosine kinases involves Ras and the Raf serine-threonine protein kinases. Immunoblotting demonstrated that B-Raf and C-Raf are

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expressed in L929 cells (not shown). Treatment of L929 cells with bFGF resulted in the activation of both B-Raf and C-Raf as measured by their ability to phosphorylate a recombinant kinase-inactive MEK-1 protein (Gardner, A.M., Lange-Carter, C.A., Vaillancourt, R.R., and Johnson, G.L. (1994) *Meth. Enzymol.* 238:258-270). MEK-1 is the protein kinase phosphorylated and activated by Raf, which in turn phosphorylates MAPK on both a tyrosine and threonine resulting in MAPK activation (Crews, C.M., Allesandrini, A., and Erikson, R.L. (1992) *Science* 258:478-480; Crews, C.M., and Erikson, R.L. (1992) *Proc. Natl. Acad. Sci. (USA)* 89:8205-8209; Nakielnny, S., et al. (1992) *EMBO J.* 11:2123-2129; Seger, R., et al. (1992) *J. Biol. Chem.* 267:14373-14381). In contrast, TNF α does not significantly activate either isoform of Raf in L929 cells.

bFGF and TNF α independently regulate cytoplasmic protein kinase cascades Fig. 27 demonstrates that 1 ng/ml TNF α has only modest stimulatory effects on MAPK activity (panel B) and 2.5 ng/ml bFGF has little or no effect on JNK activity (Panel A). These concentrations of bFGF and TNF α give maximal activation of MAPK and JNK, respectively. Co-stimulation of L929 cells with bFGF, at concentrations that show partial protection against TNF α -mediated killing, did not alter the magnitude of JNK activation in response to TNF α . Similarly, co-stimulation of L929 cells with TNF α , at concentrations capable of causing cell death, had little or no effect on bFGF stimulation of MAPK activity (Panel B). Thus, in relation to JNK and MAPK, TNF α and bFGF receptors independently regulate the activity of these two sequential protein kinase pathways in L929 cells.

Inducible expression of inhibitory and activated Ras influences apoptosis Ras activation is required for many of the phenotypic responses resulting from the activation of tyrosine kinases. Signaling by the bFGF receptor involves several different effector pathways including Ras activation. To test the involvement of Ras in the bFGF protective response, the Lac Switch inducible expression system (see Methods) was used to control the expression of inhibitory N17 Ras and constitutively activated V12 Ras in L929 cells. Fig. 28 shows the functional consequence of expressing inhibitory N17 Ras or activated V12 Ras on MAPK and JNK activation in response to bFGF and TNF α , respectively. IPTG-regulated expression of the HA epitope-tagged Ras mutants (N17 and V12 Ras) is shown in Panel D. Expression of N17 Ras significantly blunted bFGF stimulation of MAPK (Panel A), but had no effect on TNF stimulation of JNK (Panel C). With two independent clones, expression of V12 Ras did not constitutively activate the MAPK pathway, but did appear to enhance bFGF stimulation of MAPK (Panel B). V12 Ras expression also had no effect on TNF α stimulation of JNK activity (Panel C). Similar results were found with independent L929 cell clones indicating the responses were the result of specific mutant Ras expression.

Inhibition of MEK and MAPK stimulation prevents bFGF protection from apoptosis The Parke-Davis compound, PD #098059 inhibits the dual specificity protein kinase, MEK-1, which specifically activates p42/p44 MAPK (Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., and Saltiel, A.R. (1995) *J. Biol. Chem.* 270:27489-27494). PD #098059 did not inhibit JNK kinase or the activation of JNK (not shown). Pretreatment of L929 cells with PD #098059 inhibited bFGF stimulation of MAPK activity (Fig. 30A). The PD #098059 compound had no effect on TNF α -mediated apoptosis but inhibited the protective action of bFGF (Fig. 30B). Thus, MEK activation of MAPK is required for bFGF protection against TNF α -mediated apoptosis. Interestingly, the phosphatidylinositol 3-kinase inhibitor, wortmannin, did not influence the cell death response to TNF α nor did it inhibit the protective response to bFGF (not shown). Treatment of L929 cells with wortmannin had no effect on the ability of bFGF to stimulate MAPK activity. Apparently, phosphatidylinositol 3-kinase activity is not required for the action of either TNF α or bFGF on the control of the cell death program L929 cells.

TNF α induces apoptosis of L929 cells and bFGF is protective against this cell death response. Our results indicate that the activation of JNK in response to TNF α stimulation of L929 cells is not sufficient for the induction of cell death. TNF α maximally stimulates JNK activity in the presence of bFGF concentrations that are capable of protecting against cell

Recently, it was demonstrated using PC12 cells that the JNK pathway was involved in mediating apoptosis in response to serum deprivation and that activation of the MAPK pathway was protective against serum deprivation (Xia, Z., et al.(1995) *Science* 270:1326-1331). Phosphatidylinositol 3-kinase activity has also been reported to be necessary to protect PC12 cells from serum deprivation induced apoptosis (Yao, R., and Gooper, G.M. (1995) *Science* 267:2003-2006). Interestingly, the expression of N17 Ras protected PC12 cells from nerve growth factor withdrawal induced apoptosis (Ferrari, G., and Greene, L.A. (1994) *EMBO J.* 13:5922-5928). The findings indicated that N17 Ras maintained PC12 cells in a quiescent state that allowed them to survive in the absence of trophic factors. Removal of trophic factors from PC12 cells appeared to induce an aberrant proliferative response that resulted in apoptosis. Our findings using N17 Ras expression in L929 cells contrast with those in PC12 cells. TNF induced apoptosis in growing L929 cells, N17 Ras expression did not affect the apoptotic response, while V12 Ras expression significantly enhanced apoptosis.

In human B cells, crosslinking of surface IgM stimulated a host of signaling pathways including MAPK but not JNK and resulted in apoptosis (Sakata, N., Patel, H., Aruffo, A., Johnson, G.L., and Gelfand, E.W. (1995) *J. Biol. Chem.* 270:30823-30828). CD40, a member of the TNF receptor family, activated JNK while rescuing B cells from anti-IgM mediated apoptosis (Sakata, N., Patel, H., Aruffo, A., Johnson, G.L., and Gelfand, E.W. (1995) *J. Biol. Chem.* 270:30823-30828). Thus, in human B cells MAPK activation is insufficient to protect against apoptosis and signals including the stimulation of JNK are generated during a protective response. Clearly, the integration of multiple signals appears to be required for apoptosis.

Cumulatively, the results in different cell types indicate that it is the integration of multiple signals from cytokines and growth factors that determines the commitment to apoptosis. Similarly, integration of multiple signals and not a single dominant signaling pathway is likely involved in the commitment to growth or differentiation. The requirement for signal integration may allow for specific checkpoints so that cells do not die or grow inappropriately. In this regard, cell systems where specific cytokines or growth factors are added or removed are most relevant in defining the integration of signals controlling growth versus death.

The implication of our findings is that it should be possible to define signal pathways and their integration that controls apoptosis in specific cell types. As these findings are further defined it will be possible to develop strategies to selectively induce a cell type-

specific apoptotic response. Development of gene therapy, cytokine and drug treatments may be possible to selectively promote the death of undesirable cell populations in animals.

Example 31. This example illustrated the translocation of MEKK1 and MEKK2 in response to EGF and TNF α .

Swiss 3T3 cells were serum starved overnight and then treated for 10 minutes with either EGF or TNF α . Cells were fixed and stained with an antibody specifically recognizing either MEKK1 or MEKK2. Secondary FITC-conjugated anti-rabbit IgG antibody was used for staining.

The results indicated that MEKK1 was localized primarily in the cytoplasm. A weak plasma membrane staining was also evident. MEKK2 was primarily cytoplasmic with little or no plasma membrane staining.

Stimulation with EGF induced a dramatic translocation of MEKK1 to the plasma membrane. treatment of the cells with EGF did not effect the cellular localization of MEKK2. Stimulation of the cells with TNF α induced a translocatin of MEKK2 to the plasma membrane. TNF α had no effect on the cellular localization of MEKK1. Both EGF and TNFa stimulate the Jun kinase (JNK) pathway but regulate different MEKKS. EGF selectively regulates MEKK1 and TNFa selectively regulates MEKK2. The significance of this finding is the demonstration that growth factor receptor tyrosine kinases and cytokine receptors of the TNF family selectively and differentially regulate specific MEKK enzymes.

The foregoing description of the invention has been presented for purposes of illustration and description. Further, the description is not intended to limit the invention to the form disclosed herein. Consequently, variations and modifications commensurate with the above teachings, and the skill or knowledge in the relevant art are within the scope of the present invention. The preferred embodiment described herein above is further intended to explain the best mode known of practicing the invention and to enable others skilled in the art to utilize the invention in various embodiments and with various modifications required by their particular applications or uses of the invention. It is intended that the appended claims be construed to include alternate embodiments to the extent permitted by the prior art.